

Britain takes up the SGHWR

AFTER four years of debate, during which it often seemed that no decision would ever be made, the government has finally jumped off the fence, bowling over Sir Arnold Weinstock and the Central Electricity Generating Board (CEGB) in the process. For, like it or not, the CEGB is going to have to order a small number of steam generating heavy water reactors (SGHWRs) in the next few years rather than a considerably larger number of the American pressurised water reactors (PWRs) which it favours.

The remarkable thing about all this is that the decision could equally well have been made in 1972 when Mr John Davies, then Secretary of State for Trade and Industry, announced a programme of component development for the SGHWR. Other options for the short term could still have been kept open and indeed that is exactly what Mr Eric Varley, Secretary of State for Energy, has done nearly two years afterwards in saying that the Nuclear Installations Inspectorate should complete its examination of the safety of the American reactors.

Even if Mr Davies had also opted for a relatively modest scheme, Mr Varley might now be in a position to enlarge a programme to which the nuclear power industry and the CEGB would have had two years to adjust. As it is, Sir Arnold, who is head of GEC (a 50% stakeholder in the National Nuclear Corporation), is sulking and threatening to reduce considerably, or even eliminate, his company's share in the corporation. That would be embarrassing, as GEC is supposed to manage the building of the next generation of nuclear reactors in Britain and it will no doubt cause wry smiles among members of the Select Committee on Science and Technology which thinks that a shareholding of more than 30% by any commercial interest should not have been allowed in the first place. In fact it is difficult to see how the corporation could continue if Sir Arnold were to depart completely.

Ultimately, however, it is the CEGB which has to generate the electricity and its views deserve the most sympathetic consideration. It is that body, one has to bear in mind, which still has all five advanced gas cooled reactor stations waiting to be completed almost ten years after the first one was ordered. Basically the CEGB wants to order nuclear power stations in such numbers over the next decade or so that its reliance on fossil fuel is decreased, and it wants those nuclear power stations to work properly. Not surprisingly, therefore, it criticises the government's decision on the grounds that the SGHWR is untried in a commercial situation and that because of this the government is only risking a programme of some 4,000 MW to be ordered over the next four years and commissioned in the 1980s. Even the quite modest rate

of growth of electricity demand now envisaged by the Department of Energy requires 35,000 MW of new capacity by 1990, the lion's share of which will evidently be based on fossil fuels unless the SGHWR turns out to be such a success that more can be rapidly ordered after the first has become operational, perhaps in 1981.

The CEGB points out, and the government apparently agrees, that more development work will have to be done before the SGHWR can be built on even the scale that Mr Varley envisages (small units of 660 MW). The argument goes that the test reactor at Winfrith is only 100 MW and is based on technology that is already 10 years old. Parts will have to be redesigned, says the CEGB, and different materials will have to be used. But is all this really necessary? Does a new piece of functional equipment necessarily have to incorporate the most up-to-date technology wherever possible? Excessive zeal in this direction would seem to be ill advised, especially if an attempt to wring that extra bit of efficiency or cost out of the system were to cause more expense in the long run than could ever be saved. After all the suggestion that more Magnox stations should be built is only a nonstarter because of the high capital cost compared with more modern reactor designs.

What the CEGB seem to overlook is that the Canadian CANDU reactor, which is similar in many ways to the SGHWR, has been working successfully for several years and that technological cooperation between Britain and Canada is thought by both countries to be a mutually advantageous prospect. And there seems no reason why Britain should not now take advantage of the export opportunities which may still exist for the SGHWR.

100 years ago



WE rejoice to see from the tone of the replies to questions in the House of Commons on Monday by Mr. Disraeli and Lord Henry Lennox, that Government is conscious of how poorly housed some of our scientific collections are, and seems really disposed to take steps to remedy the evil. Mr. Disraeli said, in reply to a question concerning the Patent Museum, that it is not the only public institution which is suffering from want of space and of suitable accommodation. "That is now a crying grievance with respect to all our public buildings, collections, and offices. In regard to the Patent Museum, however, I am aware from a communication which I have received from my noble friend the First Commissioner of Works, that the matter is at present engaging attention." Lord Henry Lennox confirmed this by subsequently stating that he intended to propose to Her Majesty's Government a scheme which, if it were agreed to, would enable him to offer the Patent Museum suitable accommodation in the southern block of the International Exhibition buildings.



NATO and civil science

Peter J. Smith, who once accepted a NATO Fellowship because it was the only support available for work in a nonuniversity research institution in the United States, discusses NATO's involvement in civil science.

WHAT is the connection between stress and anxiety in modern society, the chemistry of insects, cosmological models, and the mooring and berthing of ships? The answer is that in common with more than 50 other science-based activities they were the subjects of well financed conferences during 1972. On the face of it, of course, there is nothing very strange about that; it is a common enough occurrence for scientists to congregate with their colleagues around the world to discuss new results and ideas, and some of them even find it useful. But there is something a little odd about it in this case, for all 56 meetings were sponsored and paid for by a single organisation which has nothing directly to do with academic science at all—the North Atlantic Treaty Organisation (NATO). The question is: why?

Scientists do pretty well out of NATO, and not just in the matter of conferences. The total budget of the NATO Science Committee in 1972 was \$4.83 million of which only \$0.93 million went on scientific meetings. Well over half was spent on the Fellowship Programme. Research grants then accounted for a further \$0.61 million, and a few specialised programmes (such as operational research, oceanography and human factors) and administration took the rest.

The mandate for all this involvement in basic science is Article 2 of the North Atlantic Treaty (1949) by which "the Parties will contribute toward the future development of peaceful and friendly international relations by strengthening their free institutions, by bringing about a better understanding of the principles upon which those institutions are founded, and by promoting conditions of stability and well-being". For the first eight years of NATO's existence the emphasis was clearly on "defence"; but in 1957 Paul Henri Spaak, the second Secretary General, made a serious attempt to widen the field to include ostensibly nonmilitary collaboration. One result was the establishment in 1958 of the

NATO Science Committee to "strengthen the Alliance and its political substance"—a form of words whose deeper meaning has apparently never been in doubt, for NATO has never made any secret of the fact that it sees its support for science as contributing, however indirectly, to its military and political aims.

In view of the often violent reaction against the military sponsorship of university science in recent years, a basically military organisation which not only publicises its involvement in civil science but also extols the virtues of it in print may appear at first sight to be either remarkably honest or incredibly naive. In fact, NATO is neither. It is not genuinely honest to be completely open about a participation which would surely be discovered in any case; and the charge of naivety would only be substantiated if it could be shown that members of the NATO Science Committee were unaware of the strength of feeling already expressed on the issue of military sponsorship. Moreover, to the extent that the sort of science sponsored by NATO furthers military aims no more nor less than it would if sponsored by an overtly civil body, financial aid from NATO as such would seem to have little relevance to that organisation's stated aims. One can only conclude, therefore, that NATO's public stance is designed to conceal motives which are altogether more subtle.

Indeed, what NATO has really been after from its large scale support of basic science—and what it has to a large extent achieved—is a sort of social respectability and prestige among a community containing elements who are decidedly touchy over the relationship between science and the military. Since 1959 over 10,000 NATO Fellows have written tens of thousands of legitimate scientific papers, each of which records gratitude for NATO support; every moderately sized scientific library in the world now contains hundreds of volumes bearing witness on their title pages to NATO's generosity; and every conference announcement implicitly proclaims NATO's apparent concern for disinterested scientific scholarship and culture. In addition, the NATO Science Committee has always gone out of its way to attract to membership established and high-ranking scientists or government scientific advisers who are able to confer their own brand of legitimacy on NATO's activities.

As might be expected, some scientists have argued that none of this matters—that given that the relevant activity is essentially nonmilitary, who pays for it is of no concern. Unfortunately, however, there is evidence that NATO's scientific involvement is

not entirely beneficial to science itself, even if motives are ignored. For one thing, although the Science Committee is always quick to point out that up to 20% of the participants in its conferences come from non-NATO countries, including the communist nations of eastern Europe, it is less ready to publicise the fact that non-NATO delegates are forbidden to attend on NATO funds. And in spite of boasts about participants from the Soviet Union and elsewhere, it is doubtful whether scientists from, say, Cuba or North Vietnam would be able to attend a NATO conference in the United States. In fact, as far as I know (and certainly up to the end of 1972), no scientist from either of these countries has ever attended any NATO conference anywhere.

Thus although scientists pride themselves on their international outlook, they are not above doing less than justice to their ideals for the sake of a quick buck. And it is because of this sort of opportunism—not to mention the skilful casuistry displayed in rationalising it—that NATO science has been given little opposition.

Few would suggest that military sponsorship is the most important problem facing civil science today. At the same time, however, this is far from saying that it is of no concern at all; and there is clearly a significant minority which feels sincerely that NATO involvement in particular is a blot on the scientific landscape. The irony is that the whole NATO sponsorship scheme is totally unnecessary, except perhaps as perceived by NATO itself. The fact is that the Fellowship programme, which accounts for about 60 per cent of the NATO science budget, is administered not by NATO but by agencies in the constituent countries (the Science Research Council in Britain, the National Science Foundation in the United States). A large proportion of NATO science money is thus returned directly whence it came (and almost all of it is repatriated to the constituent nations in one form or another), NATO's sole contributions being the use of its name and, as one cynic has pointed out, an addition to bureaucratic overheads. Since most of the national agencies concerned now have parallel programmes of their own, there seems to be little point in perpetuating NATO's involvement any longer. Alternatively, if an overtly international programme of science support is felt to be desirable, it would be far preferable to make it truly international and hand over the whole NATO scheme to some clearly nonmilitary organisation such as UNESCO. Presumably this would keep everyone happy—including NATO, if it really means what it says.

international news

NAS ban on plasmid engineering

"RECENT advances in techniques for the isolation and rejoining of segments of DNA now permit construction of biologically active recombinant DNA molecules *in vitro*. For example, techniques employing DNA restriction endonucleases, which generate DNA fragments containing cohesive ends especially suitable for rejoining, have been used to create new types of biologically functional bacterial plasmids carrying antibiotic resistance markers (Cohen *et al.*, *Proc. natn. Acad. Sci.*, **70**, 3240; 1973; Chang *et al.*, *Proc. natn. Acad. Sci.*, **71**, 1030; 1974) and to link *X. laevis* rDNA to DNA from a bacterial plasmid. This latter recombinant plasmid has been shown to replicate stably in *E. coli* where it synthesises RNA complementary to *X. laevis* rDNA (Morrow *et al.*, *Proc. natn. Acad. Sci.* in the press). Similarly, segments of *Drosophila* chromosomal DNA have been incorporated into both plasmid and bacteriophage DNAs to yield hybrid molecules that can infect and replicate in *E. coli* (Hogness, unpublished; Davis, unpublished; Boyer, unpublished).

"Several groups of scientists are now planning to use this technology to create recombinant DNAs from a variety of other viral animal and bacterial sources. Although such experiments are likely to facilitate the solution of important theoretical and practical biological problems, they would also result in creation of novel types of infectious DNA elements whose biological properties cannot be completely predicted in advance.

"There is serious concern that some of these artificial recombinant DNA molecules could prove biologically hazardous. One potential hazard in current experiments derives from the need to use a bacterium like *E. coli* to clone the recombinant DNA molecules and to amplify their number. *E. coli* strains commonly reside in the human intestinal tract, and they are capable of exchanging genetic information with other types of bacteria, some of which are pathogenic to man. Thus, new DNA elements introduced into *E. coli* might

possibly become widely disseminated among human, bacterial, plant or animal populations with unpredictable effects.

"Concern for these emerging capabilities was raised by scientists attending the 1973 Gordon Research Conference on nucleic acids (Singer and Soll, *Science*, **181**, 1114; 1973), who requested that the National Academy of Sciences give consideration to these matters. The undersigned members of a committee, acting on behalf of and with the endorsement of the Assembly of Life

In an unprecedented move, the National Academy of Sciences has called for a voluntary worldwide moratorium to be placed on an area of scientific research because of potential and unpredictable hazards to human health. A statement drawn up by a committee of eminent biomedical scientists and released by the academy this week calls for a temporary halt on two types of genetic engineering research because of the risk of infecting man with bacteria containing hybrid DNA molecules whose biological properties cannot be predicted in advance.

The academy is concerned about experiments which combine fragments of DNA from different sources to form a hybrid molecule which can then replicate in bacteria such as *E. coli*, which is normally present in the human intestine. The Committee on Recombinant DNA Molecules, whose members* have all agreed individually to renounce two types of experiments involving such techniques until the potential hazards have been evaluated, has called for a committee to be established to define the hazards and to develop guidelines under which such research should be conducted. Part of the statement is given here.

*PAUL BERG, Chairman; DAVID BALTIMORE, HERBERT W. BOYER, STANLEY N. COHEN, RONALD W. DAVIS, DAVID S. HOGNESS, DANIEL NATHANS, RICHARD ROBLIN, JAMES D. WATSON, SHERMAN WEISSMAN, NORTON D. ZINDER.

Sciences of the National Research Council on this matter, propose the following recommendations:

"First, and most important, that until the potential hazards of such recombinant DNA molecules have been better

evaluated or until adequate methods are developed for preventing their spread, scientists throughout the world join with the members of this committee in voluntarily deferring the following types of experiments:

"Type I: Autonomously replicating bacterial plasmids that might result in the introduction of genetic determinants for antibiotic resistance or bacterial toxin formation into bacterial strains that do not presently carry such determinants, or construction of new bacterial plasmids containing combinations of resistance to clinically useful antibiotics unless plasmids containing such combinations of antibiotic resistance determinants already exist in nature.

"Type II: Linkage of all or segments of DNA from oncogenic or other animal viruses to autonomously replicating DNA elements such as bacterial plasmids or other viral DNAs. Such recombinant DNA molecules might more easily be disseminated to bacterial populations in humans and other species, and thus possibly increase the incidence of cancer or other diseases.

"Second, plans to link fragments of animal DNAs to bacterial plasmid DNA or bacteriophage DNA should be carefully weighed in light of the fact that many types of animal cell DNAs contain sequences common to RNA tumour viruses. Since joining of any foreign DNA to a DNA replication system creates new recombinant DNA molecules whose biological properties cannot be predicted with certainty, such experiments should not be undertaken lightly.

"Third, the Director of the National Institutes of Health is requested to give immediate consideration to establishing an advisory committee charged with (a) overseeing an experimental program to evaluate the potential biological and ecological hazards of the above types of recombinant DNA molecules; (b) developing procedures which will minimise the spread of such molecules within human and other populations, and (c) devising guidelines to be followed by investigators working with potentially hazardous recombinant DNA molecules.

"Fourth, an international meeting of involved scientists from throughout the world should be convened early in the coming year to review scientific progress in this area and to further discuss appropriate ways to deal with the potential biohazards of recombinant DNA molecules."

Wind of change at WMO

by John Gribbin

THE World Meteorological Organisation (WMO) has acted promptly on the statement made by Dr Henry Kissinger to the sixth special session of the United Nations General Assembly calling attention to the possible implications of climatic change for global food and population policies. It has been decided to establish a "Panel of Experts on Climatic Change" with the terms of reference:

- To review present WMO activities relating to climatic change and to make recommendations on any additional steps which may be necessary to integrate these activities into a coherent programme.
- To act as the focal point for WMO activities on climatic changes, trends and fluctuations, and their implications on the natural environment of mankind and on world food production.
- To advise on any necessary measures for the coordination of these activities.
- To advise on the policy implications for WMO of decisions of other international organisations relating to the impact of climatic changes.

Members of the panel have not yet been appointed, with the exception of the chairman Dr E. Süssenger, who is a member of the Executive Committee of WMO and head of the German meteorological service.

The panel will meet as soon as possible and is requested to prepare a report in time for consideration at the Seventh Congress of WMO, next year.

At present, the WMO is active in several areas of climatic research, although these are not coordinated. Perhaps best known is the work on the physical basis of climate and climate modelling, which is being carried out jointly with the International Council of Scientific Unions (ICSU) within the framework of the Global Atmospheric Research Programme (GARP). There are several international conferences in the pipeline including one on "Long-Term Climatic Fluctuations" to be held at the University of East Anglia next August; a spokesman for the Climatic Research Unit there said last week that many participants have already accepted invitations to attend.

Other aspects of climatic research are at present being studied under the aegis of WMO by their Commission for Atmospheric Sciences (CAS), and there is also a Commission for Special Applications of Meteorology and Climatology (CoSAMC) working group looking into problems of "Climatic Fluctuation and Man". It is perhaps hardly surprising that the president of the Commission for Agricultural

Meteorology (CAGM) is about to appoint a Rapporteur on climatic fluctuations and food production, or that the WMO is preparing papers relating to climatic fluctuations for presentation at the World Food Conference in Rome in November 1974. But clearly the time is certainly ripe for one panel to coordinate all these activities, and also to relate observations by the World Weather Watch (WWW) to the WMO studies. The new panel will include experts nominated by CAS, CoSAMC, CAGM, and the Joint Organising Committee of GARP.

Among the possibilities considered by the WMO Executive Committee were to designate either the CAS Working Group on the Physics of Climatic Fluctuations or the CoSAMC Working Group on Climatic Fluctuations and Man as a panel of the Executive Committee overseeing the WMO's climatic work. But the committee decided "in view of the desirability of formulating proposals . . . for consideration at the Seventh Congress, and bearing in mind the number of constituent bodies involved . . . to establish a [new] panel of experts" with the terms of reference as outlined above.

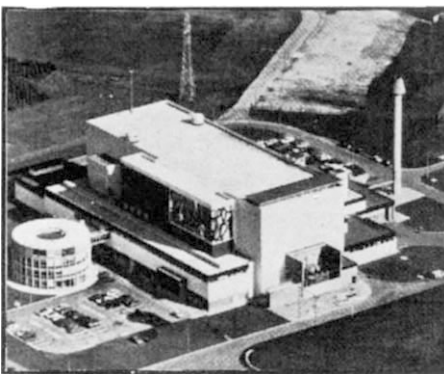
This panel will also be responsible for ensuring that WMO initiatives in this area are related to studies and work by other international bodies such as

UNESCO, the Food and Agricultural Organisation (FAO), the United Nations Environmental Programme (UNEP) and ICSU. A UNEP representative at the recent WMO Committee meeting confirmed the desire of UNEP to be associated with WMO in further studies of climatic change, and mentioned particularly the possibility of accelerating "those aspects of the WMO programme which would be of value for planning food production, especially in the developing regions". He suggested that statements of the statistical probabilities of successive years of unfavourable weather conditions would be particularly useful.

A spokesman for the British Meteorological Office welcomed this development, and said that the Met. Office accepts the WMO view that the future of our climate is an important topic for study. He pointed out that although climatic change has been receiving more attention lately, this has included publicity for some "shakily based views" ranging from claims that a new ice age is just around the corner to fears that the Earth is heading for a global desert situation. So it is certainly, in the view of the Met. Office, a good thing for an authoritative study of this kind to be carried out, and to put the various claims made about climatic change into perspective.

UK reactor choice

by Eleanor Lawrence



Winfrith SGHWR

At last the steam generating heavy water reactor (SGHWR) has been officially passed as the reactor choice for the next phase in the British nuclear power programme.

Announcing his decision in Parliament last week, Mr Eric Varley, Secretary of State for Energy, stressed five grounds on which he favoured the SGHWR.

- It will provide power reliably
- It can be ordered quickly
- The Nuclear Installations Inspec-

torate will give it safety clearance quickly

- It offers scope for British technology which should be exploited
- A 100-MW prototype at Winfrith has been operating successfully for six years and is designed to produce the operating conditions of a commercial unit.

Mr Varley plans to start with modest reactor units of around 600 MW and an initial ordering programme of not more than 4,000 MW over the next four years.

The government's decision runs counter to the strongly expressed wishes of the Central Electricity Generating Board (CEGB) in several respects. It wanted a large programme of a total of 36,000 MW using the American pressurised water reactors (PWRs).

Doubts on the safety of the PWR have been expressed both in the United States and in Britain, notably by Sir Alan Cottrell, former Chief Scientific Adviser to the Government. The Select Committee on Science and Technology was also opposed to the PWR. One of the main attractions of the SGHWR is undoubtedly its safety. Unlike the PWR there is no large pressure vessel, but a system of small-diameter pres-

sure tubes. These offer the 'leak-before-break' safety features which people feared might not occur with the pressure vessels of the PWR.

All the components of the SGHWR, except for the heavy water used as a moderator and perhaps the zirconium pressure tubes, can be made in Britain. Mr D. R. Smith, chief SGHWR engineer with the Nuclear Power Group, said that there would be few scaling-up problems except perhaps with the calandria tank surrounding the radioactive fuel elements. If the first orders were placed fairly quickly, as promised by Mr. Varley, building could start next year. Prospective sites include one at Torness in Scotland, for the South of Scotland Electricity Board (SSEB), and at Sizewell in Essex for the CEGB. Mr Smith thinks that the reactor could be completed.

The satisfactory Canadian experience with the similar CANDU reactor was probably a major factor in Mr Varley's decision. British experts who visited Canada recently were enthusiastic and close cooperation with the Canadians is expected. For the present, until the British programme is expanded, heavy water for the reactors will be imported from Canada. As soon as the SGHWR programme proves its worth, however, a British heavy water plant is envisaged, which would take advantage of the Canadian experience.

Money for old excavations

from Ian Caruana

THE Excavations Annual Reports, recently published by the British Department of the Environment (DOE) reveal that in at least one way archaeologists are failing to justify the additional cash they have been given in the past two years. In 1972 the annual government budget was in the region of £400,000; this year it has topped £1 million. One of archaeology's unceasing and justified complaints has been that the system of ministry grants for excavation never took account of the overriding need for publication, and financial austerity often made it a matter of necessity that an excavator spent almost all his time in the field just to make a living. It is, however, clear from the DOE's Reports that more money has not meant more publications. In fact while the number of digs financed directly by the DOE has risen steadily from under 80 in 1961 to over 200 in 1972 the number of reports published each year has remained more or less constant. The result is that there are now proportionally more excavations whose findings go unpublished than there were in 1961.

There is, however, one area above criticism—namely the permanent units

that are a developing feature of modern archaeology. (Their activities do not swell the DOE statistics because, although they receive a lot of government money, they also tap other sources and the DOE is quite happy to abdicate control in such cases.)

The Guildhall Museum's Department of Urban Archaeology in London is one such unit which, although set up only recently, has a good record. The results of two excavations from 1972, at Aldgate and Bush Lane House, were published last year. Four more reports are due out soon, one of which (dealing with work at the Customs House) took only five months to complete. Brian Hobley, the director, is responsible for a policy that ensures publication as quickly as possible. Each time a supervisor finishes a site he is immediately removed from the field and has to finish his site report before being given another site to run.

In addition the London unit is engaged in clearing the backlog of unpublished sites in the City. Through lack of resources, Peter Marsden (now Hobley's deputy) accumulated 70 sites which have so far yielded no published information about London's history. Now he faces a ten-year programme of publication—a feasible proposition inside a large unit with enough resources to back him up and to enable others to carry on with imminent excavation needs.

Salyut and after

from Vera Rich

ALTHOUGH the flight of Salyut 3 must inevitably be seen, in the short term, as a preparation for the joint Apollo-Soyuz programme of 1975, some of the experiments included seem directed at the longer term aim of establishing a permanent Earth-orbital station, as envisaged by Tsiolkovskii, and/or long term deep-space missions.

Accordingly, one of the major tasks of this mission is the testing of the life support systems, including the heat regulation system "in various regimes", and the regeneration of water from atmospheric condensation. The medical tests, effected with the multipurpose "Polinom-2M" apparatus, include the investigation of haemodynamics (the state of the circulation and the "rate of propagation of pulse waves along the arteries"), the ventilation of the lungs, and the collection of samples of exhaled air for subsequent laboratory analysis. It is intended to use the respiratory data to calculate the energy consumption in performing various tasks under weightless conditions—results which could be of considerable importance in planning the nutritional

requirements of a fully operational long term station or mission.

What these tasks are, either in the present mission or in future plans, is so far not entirely clear. The TASS reports, as ever, are tantalisingly brief, stating in this case, that the cosmonauts have "begun to test the possibility" of manufacturing hitherto unknown substances. A hint from the mission control station suggests that these may include steel "lighter than wood", and glass/metal alloys. In view of this brevity, speculation would seem fruitless at present.

Nevertheless, this type of experiment does suggest the Tsiolkovskii concept of a fully operational and self-maintaining orbital station, utilising the space "environment" for technology as well as research work. Although Tsiolkovskii's belief that the creation of such a station would be a necessary preliminary to any lunar or deep-space mission has been superseded by subsequent technology, Tsiolkovskii's name still exerts a considerable charismatic effect on the Soviet space planners. It is perhaps significant that, in a *Pravda* "background article" to the present flight, Candidate of Sciences N. Pisarenko of the Institute of Space Research of the Soviet Academy of

Sciences, discussing the radiation hazards of long term flights, states that primary cosmic radiation of galactic origin would, in the present state of the art, form a definite barrier to any manned Mars mission. He notes that "ways of overcoming this barrier" are being investigated, including "study of the self-shielding, restorative functions of the human organism and possibilities of intensifying them by the use of pharmacological preparations", as well as improvement of the shielding of the spaceship itself. For orbital stations, however, the hazard is considerably less, since according to estimates obtained from the data of Kosmos satellites, at the altitudes at which space stations operate (apogee of up to 500 km) cosmic radiation of solar origin is virtually absent and galactic primary cosmic radiation reduced by some three or four times in comparison with the deep-space value.

Both types of protection received an unexpectedly severe test when a solar flare occurred during the flight. According to TASS (July 15) "some physicists" wished to recall the cosmonauts. But it was decided that the Earth's magnetic field would protect them from the worst of the radiation, and they were instructed to use their antiradiation drugs.

MRC restructuring

by Peter Newmark

FROM September 1, 1974, substantial changes in the research boards that serve the Medical Research Council (MRC) will take effect. The Tropical Medicine Board is to be retained but both the Biological and Clinical Research Boards will be scrapped. In their place there will be three new ones—the Neurobiology and Mental Health Board, the Cell Biology and Disorders Board and the Physiological Systems and Disorders Board. It is the task of each board to advise the council on policy and to initiate and support research within its designated area of scientific objectives. In addition each board has the responsibility of maintaining research in and deciding the level of support needed for each of the disciplines or specialties that it encompasses.

As well as the four boards there is also to be an Environmental Medicine (Research Policy) Committee. This will cover all aspects of environmental, occupational and social medicine. The fact that these areas will include subjects (for example radiological protection) that are also of concern to the boards is recognised by ruling that the committee will allocate its funds as far as possible through the boards. Similarly each board will be able to use its funds for subjects covered by the committee.

The new boards seem to have a degree more power than previously. Whilst the council still carves the cake, it will be largely for the relevant board to allocate its own slice. One can hardly help noticing that the total number of board members has expanded much faster than the money available for them to share out (not forgetting that by 1975–76 about 25% of the MRC's grant-in-aid will be transferred to the Department of Health and Social Security and the Department of Employment to enable them to commission contract research to be carried out by the council).

The main objective behind the new MRC structure is clearly a step, or at least a nod, in the direction of Rothschild thinking. Instead of a horizontal structure dividing clinical from basic research each board is now vertically structured.

Erratum. In the article "Foetal research aborted in the United States" (*Nature*, July 12) the last sentence of paragraph 3 should have read "... research on fetuses *in vivo* has already produced some valuable conclusions and its banning is seen by some as shortsighted and ill-founded."

Lecturers lectured

FOUR lecturers at the Hebrew University in Jerusalem have been fired and another 12 transferred to new posts for poor teaching. Their achievements as teachers, as those of 220 colleagues, were evaluated on the basis of a survey carried out among several hundred science students at the Hebrew University by the Jerusalem-based Institute for Applied Social Research. The lecturers were graded on a five-point scale, with one point going to the worst and five to the best. Taken into account were student answers to questions such as: "Is the material usually presented in an interesting manner?"; "What attitude does the lecturer have towards relevant comments and questions?"; and "To what extent does the lecturer add to the information already available in textbooks?"

Analysis of the replies received has enabled the university to advise even relatively effective lecturers how they can overcome weak points. Professor Yitzhak Marcus, Chairman of the Curriculum Committee in the Natural Sciences Faculty of the Hebrew University, explains that the survey was not undertaken "to punish" lecturers.

New ways with fuels

by Allan Piper

IT was encouraging to hear original ideas given an airing recently in London where the Institute of Physics had arranged a meeting on "Novel physical methods of winning, burning and conserving fuels". Plants could be harnessed to produce hydrogen, said one participant, and another introduced the futuristic concept of mechanical miners. Most of the ideas are already well developed, but inevitably the 'establishment' found them 'uneconomical', and by implication not worth developing.

One topic was the potential use of solar energy through photosynthetic conversion to energy-rich plant material, which can then be converted by pyrolysis to alcohol, gas, oil and solid fuels. Dr O. D. Hall (Kings College, London), a botanist, pointed out that the amount of solar energy arriving at the Earth in three hours can meet the world's power demands for a year.

Photosynthesis in many economic crops is relatively inefficient. But in some other plants a more photosynthetic system, C₄, operates. Attempts to introduce the C₄ system into economic crops are in progress, together with a programme of plant breeding

aimed at developing plants which are more efficient and will produce specific desirable products. Hydrogen gas, a likely fuel for the future, can also be produced by photosynthesis, said Dr Hall. Under certain growing conditions algae can produce hydrogen, and model chloroplast-membrane systems have generated hydrogen in the laboratory.

If sunlight could be converted with an efficiency of 10% (compared with 0.2% naturally) only 1.5% of the land area of the United States would be needed to provide the total energy requirement of that country. Sunnier South Africa and Australia would need less still and even densely populated Britain would need to use only 9% of her land area.

Dr Hall stressed that an integrated approach using a variety of organisms, not only green plants, would be needed but that it would then be possible to harness all the sunlight between wavelengths of 400 and 900 nanometres.

Participants who found Dr Hall's ideas too revolutionary found little consolation in the next lecture. Professor M. W. Thring (Queen Mary College, London) said that much of the world's coal resources can never be tapped if we continue to use human miners. He introduced the concept of 'telechurics' hands at a distance. Already such automation has been used to service nuclear reactors and on the Moon. But the machines for mining coal would have to perform all the functions of a human miner. Professor Thring believes that expenditure of less than £100 million could develop such mechanical miners within 10 years; they would be operated by one worker from the safety of a control room.

But whatever we burn, we must learn to do it more efficiently. Professor F. Weinberg (Imperial College, London) explained that natural burning is a very inefficient process. Extremely low concentrations of combustible material in air—'lean mixtures'—cannot be burnt using conventional methods of combustion. But the efficiency of burning can be improved by preheating mixtures, said Professor Weinberg, thus allowing much leaner mixtures to be burnt. Useful supplies of energy could be extracted from waste gases, which at present are allowed to escape as pollutants into the atmosphere.

Professor Weinberg also discussed how electric fields could be used to regulate the behaviour of various pollutants within a flame, such as carbon or lead oxide smokes. Pollutants could then be induced to deposit in a particular position, on an electrode for instance, and particles could be manipulated, allowing control of their development and consumption in the combustion zone.

Business: multinationals in the news

by Roger Woodham

A COMMISSION on Multinational Corporations is called for in a recent report to the Economic and Social Council of the United Nations (UN) by a "group of eminent persons". The group was set up by the council to study the "impact of multinational corporations on development and on international relations", and during its deliberations it questioned some 46 witnesses including Mr. Ralph Nader, Mr. Altiero Spinelli (a member of the Commission of the European Communities) and Sir Val Duncan (Chairman of Rio Tinto Zinc).

The report makes it clear that the primary responsibility for the behaviour of multinationals rests with individual governments, which should be backed up by action at the international level to promote cooperation and harmonisation. The commission, the group envisages, will be made up of experts acting in their individual capacities and assisted by an information and research centre within the UN Secretariat. Some sort of general international agreement is the eventual goal (perhaps something like the General Agreement on Tariffs and Trade, GATT).

Particular concern is shown in the report about the effects of multinationals on developing countries, in many of which there is "widespread concern over foreign control of key sectors of the economy". The group thinks that host countries should clearly define the areas in which they are ready to accept foreign investment and that developing countries, especially, should be encouraged to retain ownership of their natural resources or control of the use of them. More

joint ventures between multinationals and individual governments may be the answer, with capital being made available by international financial institutions.

On the technological side, the report reiterates what others have said before, namely that the most up-to-date technology is not necessarily the most appropriate for a developing country. One of the group's suggestions is that host countries set up centralised negotiating machinery to deal with all aspects of proposals for foreign investment, the nature of the technology included.

The group thinks that the matter of multinational corporations needs to be discussed at least once a year by the Economic and Social Council. And in something of an understatement the report declares that "their activities are not *per se* geared to the goals of development".

● Hoechst UK, British subsidiary of the German chemical giant Farbwerke Hoechst, formally opened its new pharmaceutical laboratories at Milton Keynes last week. With a budget of £1 million a year the new laboratories are the latest in a series that Hoechst has been setting up throughout the world in the past six years—the others are in the United States, Japan and India. The emphasis at Milton Keynes is, however, to be on development and the eventual full complement of 150 scientists will work on diagnostics and the development of possible new drugs rather than search for new chemical entities.

Hoechst has become particularly sensitive to the strong feelings that surround the use of animals for testing drugs because on two occasions last year a group calling itself the Band of Mercy attempted to burn down the laboratories as they were nearing com-

pletion. It failed, but the conspicuous presence of uniformed gentlemen from a private security company and the neatly rolled fire hoses near hydrants showed that the company is taking no further chances. Dr J. Coombes, head of Hoechst pharmaceutical research in Britain, made a special point of saying that "we would, of course, prefer to avoid the use of animals, with all their disadvantages, and we try to do this wherever we can, but in most cases it is just impossible." Safety tests on animals are required by law under the Medicines Act, he added.

First aid for the Aral Sea

from our Soviet Correspondent

UNTIL the grand, often publicised schemes for diverting the North Siberian Rivers Ob' and Yenesei so that instead of discharging into the Arctic they will irrigate the steppes of Central Asia, can at last be brought into effect, first aid measures are needed to save the Aral Sea.

This small inland sea, fed by the waters of the Amu-Darya and Syr-Darya was first hydrographically surveyed by a military expedition in 1848-9 (as part of the drive towards India), and has in the subsequent century and a quarter fallen some 3 m in level, because of the use of the rivers for irrigation.

The Aral basin contains more than half of the irrigated lands of the Soviet Union, 90% of the cotton crop, 40% of the rice and considerable quantities of fruit and vegetables. It is not, however, a lack of water in absolute terms that is the trouble, but a piecemeal use of what there is. During the spring floods the irrigated settlements are in considerable danger from flooding, and the only remedy at present is to divert the floodwaters to go to waste in the deserts.

The solution proposed is simple—a system of 13 dams to be built on the two rivers to contain the floodwater, distributing it for irrigation as required with the remainder being discharged into the sea. The surplus irrigation water will be collected and reused for irrigation and for leaching salts out of the soil, a method already in use in many of the Soviet Central Asian cotton farms.

The methods seem standard enough, but the publicity given to it and to its initiator, a certain Konstantin Rakitin (meriting a page and a half of a Novosti press release), would suggest perhaps that the prospect of a spectacular future solution—the diversion of the great Siberian rivers—has until now led to a neglect of the necessity for short term conventional measures.



New Hoechst laboratories

correspondence

Newton and Kepler

SIR,—The exchange of letters between J. Herivel and D. T. Whiteside (*Nature*, April 19, 634) arising from Herivel's review of my *Introduction to Newton's Principia* (*Nature*, 247, 163-4), hinges primarily on my alleged interpretation of an article by Whiteside (1964) and only secondarily on the substantive issue: Isaac Newton's possible knowledge of Kepler's law of areas before, 1676 or so, or even later.

When Whiteside's article appeared in 1964, I had already been engaged for several years in wholly independent research on the development of planetary astronomy before the *Principia* and on the source of Newton's knowledge of Kepler's laws. One of the central topics of my investigation had been the use of one of three then-current variant substitutes for (or approximations to) the area law—associated with the names of Bullialdus, Ward and Mercator. As I read through Newton's early manuscripts and book annotations, I was struck by the fact (it would have been impossible not to have been!) of the obvious and conspicuous lack of any early documentary reference by Newton himself to the law of areas—there is none dating from the period up to the famous exchange of letters between Hooke and Newton in 1679–80.

When Whiteside's brilliant article appeared, I temporarily laid aside my own book-length manuscript, but the postscript (page 137) to Whiteside's article refers to this work of mine. Accordingly, Herivel's remarks as to whether my opinion is or is not simply a correct interpretation of Whiteside's article may be seen to be irrelevant.

The substantive point at issue is not whether Newton may possibly have encountered the law of areas before 1676 (at which time he may very well have read the statement of the law in Mercator's treatise). It is rather whether at any time earlier than the end of his exchange of letters on motion with Hooke, in 1678–79, Newton consciously gave to this law any serious consideration as a possible basis of physical principles, or as an accurate (or most accurate) descriptive statement of the variations in planetary orbital speeds, or even as a major or significant element in considering planetary motions of the same order of

importance, say, as the elliptical orbits or the harmonic law. On the basis of ordinary canons of historical evidence, and to give my view the most accurate expression possible, the footnote in my *Introduction*, which is the occasion for these letters may be (in part) expanded and rewritten more fully so as to read:

"There is no documentary evidence that Newton was consciously aware of Kepler's law of areas (much less that he considered this law in any significant manner) prior to 1676, when he might well have encountered it in Mercator's *Institutionum Astronomiarum Libri Duo*. At the end of his correspondence on motion with Hooke in 1679–80, or possibly soon thereafter, but at least by 1684, he used this law to solve the central problem of elliptical planetary motion, shown to result from the action of a centrally directed inverse-square force on a body with a component of linear inertial motion."

Yours faithfully,

I. BERNARD COHEN

Cambridge, Massachusetts

Family planning

SIR,—Poor parents in poor countries do not readily adopt family planning methods even when these are available to them, because they do not want to have small families. They want large families for various reasons, many of which are economic; children provide free labour on family farms, security in old age and so on. Economic motivations can be changed by economic means. Under present circumstances a substantial reduction in the rate of population growth appears to depend on widespread economic improvement: but in many developing countries widespread economic improvement does not seem feasible unless there is a substantial reduction in the rate of population growth.

The problems are familiar, but they may not be insoluble. In many parts of the world the parents' desire for large families seems to represent to a great extent a desire for sons. If such parents could choose to have sons rather than daughters they would probably do so; if a cheap and simple method existed which increased the chances of having sons rather than

daughters, it would probably be readily adopted. The total number of children needed in order to obtain any given number of sons would be reduced. And in the long term, the shortage of women of child-bearing age would lead to a further reduction in the birth rate.

Human sperm bearing X and Y chromosomes can be separated *in vitro* on the basis of their differential motility. Y-sperm, which are male-determining, have a greater ability to penetrate an interface between a less viscous and a more viscous fluid and also out-distance X-sperm by swimming faster in a fluid of relatively high density and viscosity¹. It is not inconceivable that some method could be devised whereby a suitable viscous solution could be introduced (for example within a capsule) into the female genital tract in such a way that after sexual intercourse the X-sperm were selectively retarded and the chances of conceiving a male child were increased.

The idea that the ability of parents to choose to have sons rather than daughters could lead to a substantial reduction in the birth rate, especially in countries with high rates of population growth, has been proposed before²; the recent findings on differential sperm motility suggest that this idea should now be taken seriously.

If a suitable method could be developed and if it were adopted on a wide scale, the increased proportion of males in the population would undoubtedly create new problems, some of which are easy to imagine. But what are the imaginable alternatives? In India, for example, in spite of a well-established, government-sponsored family planning organisation and mass sterilisation programme, the population is now increasing by one million every twenty-five days.

Yours faithfully,

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¹ Ericsson, R. J., Langerin, C. N., and Nishino, M., *Nature*, 246, 421–424 (1973).

² Postgate, J., *New Scientist*, 12–16 (April 5, 1973).

news and views

What does BL Lac?

MANY extragalactic objects—the nuclei of some galaxies, quasistellar objects, N systems and objects of the BL Lacerta type—contain very small components which vary in optical light, and in radio flux. The variations take place on time scales which vary from days or weeks up to many years. Objects with a wide range of redshifts are involved, and of course until recently no lines had been identified in objects of the BL Lac type.

Radio astronomers using very long baseline interferometers have measured very small angular sizes 10^{-3} – 10^{-4} arc second in some sources, and apparent changes in angular structure have been seen in several of them. The time variations which occur either in flux or in angular size, when interpreted using distances obtained from redshifts (that is on the cosmological assumption), have led to various apparent difficulties in interpretation.

The very small sizes set by the light travel time and the very large optical fluxes set by the large redshifts lead to immense photon fluxes, and apparent contradictions involved in the interaction of the photons with the electrons which must generate them. This problem, originally raised by Hoyle, Burbidge and Sargent in 1966, has been argued about, skirted around and sometimes denied.

In cases where one has angular size measurements and radio spectra which turn over, suggesting synchrotron self-absorption, detailed calculations can be made (see for example Jones, O'Dell, Stein and Burbidge *Astrophys J.*, **188**, 353; 1974). If flux variations in such radio sources are explained in terms of a simple expanding cloud model, it is found that for all sources with large cosmological redshifts and rapid variations the expansion must be highly relativistic. This means that enormous energies of the order 10^{50} erg or greater are released in frequent outbursts, part of the energy being associated with relativistic expansion against the surrounding medium. Because of the short time scales the cumulative energy problem is very severe.

In sources in which angular sizes seem to have varied, the observations were first thought to indicate relativistic motion of components—separations of doubles, and so on—at relativistic speed. Thus, there was talk of apparent superlight velocities. By now the difficulty and ambiguities of interpretation of the radio data have led some people to discount models of this type, and models in which it is supposed that several small spatially separated components are varying independently are now in vogue. These too have their difficulties.

All of these apparent problems raised by such sources stem, however, from the great distances implied by the redshifts. For comparatively close objects, such as the radio galaxy NGC1275, low and entirely reasonable optical and infrared radiation densities, non-relativistic motions and lower energies are required. Thus, for some time, either the unpopular view that the redshifts are not measures of distance, or the notion that nobody really understands the physics of the sources has been the basis of the two approaches taken by students of the subject.

Until recently the BL Lac objects which show very rapid flux variations and all of the other continuum energy properties of, for example, QSOs, could not be brought into the debate because no redshifts had been measured.

Now within the space of three months redshifts for two of them, BL Lac itself (Oke and Gunn, *Astrophys. J. Lett.*, **189**, L5; 1974) and PKS0735+178 (Carswell, Strittmatter, Williams, Kinman and Serkowski, *ibid.*, **190**, L101; 1974) have been published. What is one to make of them? The strongest claims have been made for BL Lac. Full publicity has been accorded to the view of Oke and Gunn that BL Lac is a QSO embedded in an elliptical galaxy with a redshift $z=0.07$. Discounting for a moment the fact that the paper and the news releases (which many scientists have read more carefully than the paper itself) appeared on April Fools' Day, how seriously should one take this claim? Earlier arguments of different kinds by Adams (*Astrophys. J.*, **188**, 463; 1974) and by Wlérick, Michet and Lelièvre (*C.r. hebdomadaire Acad. Sci., Paris*, **278**, 245; 1974) in which it has tacitly been assumed that a normal galaxy does underly the nonthermal object, have led to distance estimates which range from 130 Mpc ($z=0.022$) to 420 Mpc ($z=0.07$). The Oke-Gunn result rests on scanner observations of the outer parts of the object and it is claimed that weak absorption features, not all of which are identified, are those expected from stars in a normal elliptical galaxy. The problem is that the observation is very difficult, the features are weak and the reality of some, and perhaps all of the absorption lines, is in doubt.

What can be learnt from the other BL Lac object PKS0735+178? In this investigation the authors have made no claim to have discovered a stellar component. The redshift rests on two comparatively sharp absorption features which they attribute to Mg II, based on the separation between the two components of the doublet. These are not likely to be due to stars, otherwise the two components would be smeared into one broader line. The absorptions are more like those arising in gas clouds which are often seen in high-redshift QSOs. The object is variable in optical light and in polarisation on time scales less than a month.

Thus, on the face of it, these first two BL Lac objects in which spectroscopic features have been found seem to be different. One may have its source in a normal galaxy with a lineless QSO (a contradiction in terms of the original definition) embedded in it. The other, as far as is known, is a nonthermal continuum object with QSO-like absorption in it.

Should these interpretations be taken seriously? The answer is that one should be exceedingly cautious at this point. The most important thing is to check the BL Lac observations using independent and, if possible, superior equipment. It would not be at all surprising, despite the ballyhoo and the claims, if they did not survive. It is interesting to see how easily theoreticians tend to accept this result at face value, when compared with the way they looked sceptically at the original gravitational wave results.

What bearing do these results have on the problems mentioned earlier? If BL Lac is at a distance of 420 Mpc, then as Oke and Gunn point out, the luminosity and rapid variations of the central object give rise to all of the apparent difficulties associated with understanding the radiation properties of the large-redshift QSOs. The same can be said for PKS0735+178 which has an even larger redshift ($z=0.424$). At the same time the results do not provide direct evidence against the possibility of noncosmological redshifts. If it is supposed that a QSO is embedded in a galaxy, it must be proved that each component has the

same redshift if the noncosmological hypothesis is to be disproved. In BL Lac only the redshift of the outer part has been observed. The centre is excluded. This argument is the converse of that used by Kristian (*Astrophys. J. Lett.*,

179, L16; 1973) who, having observed QSOs whose redshifts had been measured and which have fuzz around their images, assumed, without proof, that these were galaxies.
GEOFFREY BURBIDGE

Three abundance classes of messenger RNA

GENE expression has both a quantitative and a qualitative component. It is important to know not only which genes are expressed but also how many transcripts of each are present. On page 199 of this issue of *Nature* Bishop, Morton, Rosbash and Richardson show by two independent methods that in HeLa cells approximately 1% of the single copy DNA, enough to code for about 35,000 different average-sized proteins, is transcribed into cytoplasmic messenger RNA which may be isolated by affinity chromatography on oligo (dT) cellulose. These mRNA molecules are present in three distinct frequency classes. About one fifth of them are transcribed from a very few (about 17) sequences but are present in great abundance (about 10^4 copies each per cell). One half of the mRNA is derived from a very large number (about 35,000) of different genes but individual mRNA sequences are present at low concentration (about 10 copies per cell). The remainder of the message population derives from an intermediate number of genes (about 350) and each is represented by approximately 450 copies per cell.

The kinds of experiment from which these numbers are derived involve purification of cytoplasmic mRNA by affinity chromatography on oligo (dT) cellulose. Although this method does not give a total message preparation (for instance histone mRNA will not be present), it seems likely that it will contain the bulk of the mRNA. Highly radioactive DNA complementary to the purified mRNA (cDNA) is synthesised by viral reverse transcriptase for use as a probe in nucleic acid hybridisation reactions. Bishop *et al.* performed two types of hybridisation reaction with their antimesage cDNA—one using total DNA in excess and one using purified mRNA in excess. The DNA-driven reaction shows that cDNA prepared from total cytoplasmic message hybridises to both repetitive (about 10%) and non-repetitive (about 70%) components. A control using purified radioactive HeLa single copy DNA instead of cDNA defined a rate constant for the renaturation of HeLa unique sequence DNA which is nearly identical to the rate constant determined for the second transition of the cDNA reaction, providing convincing evidence that cDNA is reacting with unique sequences. These results suggest that most of HeLa cytoplasmic message is transcribed from unique DNA but a small fraction (about 15%) derives from a class of intermediate repetitive sequences.

The rate of the RNA-driven hybridisation reaction between cDNA and mRNA template in excess is determined by the relative concentration as well as by the absolute number of different mRNAs in the total preparation. If all mRNAs are at equal concentrations then the reaction will have a single transition with a rate constant determined by the number of reacting species. But if some RNAs are present in greater amounts than others they will react faster and show up as an early transition or step in the progress curve of the reaction. The actual curve relating extent of cDNA hybridised to the R_{0t} (product of initial mRNA concentration and time) shows three such transitions suggesting at least three major frequency classes in the mRNA population. In order to interpret these curves, a kinetic standard of known sequence reiteration frequency and molecular weight is required. Reaction between excess rabbit haemoglobin α chain mRNA or a mixture of α and β

chain mRNA and homologous cDNA provides such a standard. A further requirement for estimating numbers of gene transcripts is a measurement of the number-average molecular weight of HeLa mRNA which was determined at 600,000 daltons—about three times the molecular weight of haemoglobin α chain mRNA. Dividing the observed $R_{0t_{1/2}}$ for each transition by the $R_{0t_{1/2}}$ of the kinetic standard corrected for the difference in molecular weights gives an estimate of the number of gene sequences contributing transcripts to that frequency class.

These calculations are necessarily approximate since they rely on unverified assumptions such as the general applicability of a number-average molecular weight determined for the total mRNA population to different subsets of that population and uncertainty about the contribution of repetitive gene transcripts to each of the three frequency classes. Furthermore, it is not clear at present whether all poly(A)-containing RNA is transcribed by reverse transcriptase with equal efficiency. In the worst case an entire frequency class might not be transcribed at all and clearly would not be detected by hybridisation. If on the other hand there were only slight variations in transcription efficiency this would not matter particularly since the rate of hybridisation depends on the concentration of cold driver, either RNA or DNA, and not on the concentration of cDNA. What might be mistaken though would be the relative amount each frequency class contributes to the total population since these are determined by the plateau values and not by the reaction rate.

A third type of experiment using purified ^3H -labelled single copy HeLa DNA instead of cDNA as the probe in an mRNA excess reaction allowed a totally independent estimate of the proportion of the genome transcribed to give cytoplasmic message. Both the cDNA and the purified single copy reactions led to the conclusion that about 1% of the unique sequence is represented in mRNA and allow some weight to be attached to this estimate.

This work represents a significant advance in the understanding of both gene expression and in the likely numbers of different sequences involved. But many intriguing questions remain. Do HeLa cells really synthesise 35,000 different proteins or do some unique sequence transcripts not code for proteins? Are HeLa cells expressing their maximum genetic potential or is even this large amount of information only a small part of their true capacity. In this regard it is noteworthy that a *Drosophila* cell line seems to express an order of magnitude fewer sequences (4,000) in cytoplasmic mRNA and it may well turn out that HeLa cells are atypical in the amount of information found in cytoplasmic message. The existence of three frequency classes of mRNA, most of which derive from single copy DNA sequences, clearly focuses attention on the differential control of mRNA synthesis and degradation. It may be that differential rates of synthesis are responsible for variation in the steady state level of particular mRNAs but equally likely at present are differential rates of degradation, for which there is already evidence in HeLa cells. Finally, it is clear that a cell contains different abundance classes of protein and it would be interesting to know to what extent this reflects the frequency classes in mRNA.

PETER J. FORD

DNA of hepatitis B virus

SIGNIFICANT advances in research on hepatitis B virus have been made recently by groups at several centres in the United States. Experiments by Hirschman and his colleagues (*Lancet*, i, 1099; 1971) first indicated the presence of a DNA polymerase activity in association with hepatitis B antigen. Crude pellets of antigen, obtained by high speed centrifugation of sera from a few patients with clinical and histological evidence of hepatitis, were found by electron microscopy to consist largely of the small pleomorphic 16–25 nm spherical particles of hepatitis B antigen, although the larger 42 nm spherical particles and tubular forms of varying lengths were also found. These preparations were found to stimulate the incorporation of tritiated dTTP into an acid-insoluble product in the presence of all four deoxynucleoside triphosphates, albeit at a low level. The reaction was considered to be dependent on an endogenous RNA template or primer since pretreatment of the samples with RNase abolished their activity. The reaction was, however, stimulated by the addition of poly (dAT) and not by poly (rA)-oligo (dT), as are the known RNA-dependent DNA polymerases.

Kaplan *et al.*, (*J. Virol.*, 12, 995; 1973) have reported the finding of a DNA polymerase activity in plasmas positive for hepatitis B antigen, selected because of their relative rich content of the 42 nm double-shelled spheroidal form of the antigen. DNA polymerase activity was associated with the core component of the 42 nm particle after removal of the outer surface antigen coat by treatment with the non-ionic detergent Nonidet-P40 (NP 40). Surprisingly the enzyme seems to function in the absence of any exogenous template. The incorporation of tritiated dTTP was dependent on the presence of all four deoxynucleoside triphosphates and magnesium ions, suggesting that a DNA or RNA template within the core probably directs the synthesis of DNA. The properties of the enzyme product were further assessed using ³H-dTTP as the trace label. Digestion with pronase followed by phenol extraction resulted in the appearance of about 20% of the acid-precipitable label in the aqueous phase. This extract possessed a buoyant density of 1.71 g cm⁻³ and a sedimentation coefficient of 110S, which was reduced to 15S after incubation with sodium dodecyl sulphate. Rate zonal centrifugation confirmed the close association of the polymerase with a proportion of the 42 nm antigen particles, within which the template molecule lies in a protected state. No enzymatic activity was found in highly purified small (16–25 nm) hepatitis B surface antigen particles. Kaplan *et al.* concluded that if the DNA polymerase is a virion enzyme and if the reaction is DNA dependent (as was suggested by inhibition of the reaction by actinomycin D and daunomycin), then the enzyme would be unique because similar virion enzymes have not been described so far.

Kaplan and his colleagues (*Nature*, 249, 762; 1974) went on to study hepatitis B DNA polymerase activity during the course of post-transfusion hepatitis. The polymerase activity was detected later than hepatitis B surface antigen but before serum transaminase levels were elevated so it was concluded that polymerase activity was related to viraemia and not to virus-induced liver damage. Krugman *et al.* (*New Engl. J. Med.*, 290, 1331; 1974) also concluded, after the examination of serial samples of serum from volunteers exposed to the MS-2 strain of hepatitis B virus, that DNA polymerase activity seems to identify the period of peak replication of hepatitis B virus.

Further observations on the nature of the polymerase and the product of its reaction were reported recently by Robinson of Stanford University School of Medicine at a seminar held at the London School of Hygiene and

Tropical Medicine (full details will be published in the *Journal of Virology*). Hepatitis B surface antibody removed the enzyme from the reaction mixture in the absence of the detergent NP 40, but in the presence of NP 40, which exposes the core, the enzymatic activity was found in the supernatant and could only be precipitated by the core antibody and not by the surface antibody. As expected from these results, the addition of purified 16–25 nm surface antigen particles effectively competed with the surface antibody and blocked the precipitation of the enzyme-containing particles in the absence of the detergent, but after treatment with NP 40 the purified small particles did not block the precipitation of the core by the core antibody. The DNA polymerase activity, after treatment of the antigen preparations with the detergent, was found at a closely similar position in a sucrose gradient as the 28 nm core particles. The 110S structure was found to be associated with the core by immunoprecipitation with hepatitis B core antibody. Advantage was taken of these two latter observations to provide a sensitive test for core antibody in sera of patients and carriers by using the DNA polymerase and the radioisotope-labelled DNA reaction product as markers for the core.

The initial finding that the DNA polymerase activity, associated with the core particle, does not require added DNA or RNA implies that the core contains nucleic acid which served as a primer template for the reaction. A major advance with this work has been the observation by electron microscopy of circular DNA molecules by shadow casting. Briefly, the procedure involved concentration of the 42 nm hepatitis B antigen particle by centrifugation followed by purification by repeated equilibrium centrifugation in sucrose density gradients. The preparations were exposed to DNase in order to eliminate free DNA. After treatment with the detergent NP 40 the cores were isolated by rate zonal sedimentation and treated again with DNase. After further centrifugation the core pellets were disrupted with sodium dodecyl sulphate to release any nucleic acid. Incubation with DNase 1 before electron microscopy removed all of the circular molecules, whereas RNase had little effect. The smooth, open configuration of the molecules suggests that they are double stranded and additional molecules of single stranded nucleic acid were not found after spreading in formamide.

In other experiments, dissociation of the core particles with lithium thiocyanate again revealed small circular nucleic acid molecules. The circular structures were not supercoiled and Robinson suggests that this appearance may be due to an open circular conformation with a nick in one of the two strands. Size distribution analysis give a mean length of the circular molecule of 0.78 μ m corresponding to an estimated molecular weight of the DNA of about 1.6×10^6 . This is smaller than double stranded DNA found in any known 'complete' virus and it is similar in molecular weight to adeno-associated virus. The thermal denaturation kinetics of the isolated DNA confirmed its double stranded nature and gave a result consistent with a G:C content of 48 to 49%.

This is a somewhat unexpected finding in view of the similarity to the base composition of mammalian DNA, and if this is representative of part or the whole of hepatitis B virus genome it is more consistent with those viruses possessing the ability to integrate their own genome into that of their host. Furthermore, it is difficult to envisage that this molecule is capable of coding for more than five or six proteins and would therefore almost certainly not contain sufficient information to satisfy the requirements both for active virus replication and the apparent complexity of the surface determinants on the particles associated with hepatitis B antigen. It is also difficult to imagine the active association of the enzyme and template within the confines of the core of the 42 nm particle. The

possibility of their segregation as components of different morphological forms, in a manner perhaps similar to the Fraenkel-Conrat covirus model for some plant viruses which require two or more particles to produce infection (a concept which was extrapolated to human hepatitis type B (Zuckerman, *Vox Sang.*, **19**, 304; 1970)) cannot yet be ruled out. There is also the possibility that the circular DNA is derived from a defective virus, and infectious particles with larger DNA molecules, which have not yet been detected, might be present. Alternatively, a helper virus might be required for replication (Kassanis, *J. gen. Microbiol.*, **27**, 477; 1962) and, although a second or helper virus has not yet been identified, such models for hepatitis have been proposed (Zuckerman *et al.*, *Nature*, **214**, 606; 1967).

The findings just reported by Robinson clearly represent one of the most significant steps forward in hepatitis research, but further work is required to establish conclusively the nature of the DNA polymerase template, to identify the enzyme as viral and not host specific and to clarify some of the other puzzling characteristics of the intriguing hepatitis B virus.

ARIE J. ZUCKERMAN
COLIN R. HOWARD

r and *K* rodents in Costa Rica

from our Animal Ecology Correspondent

THE demographic strategy employed by a species population is a function of, and is dependent on, the ecological complexion of its environment. This affirmation of the status of the population led MacArthur and Wilson (*The Theory of Island Biogeography*, Princeton University Press, 1967) to underscore and draw attention to the significance of *r* and *K* selection. In seasonally fluctuating environments it is argued that populations will seldom achieve a density corresponding to the carrying capacity (*K*) and so intra-specific competition for food and other resources will be low. Natural selection will favour those genotypes that produce large litters of early maturing young. In environments that lack a marked seasonal boost in productivity, that is, with *K* remaining constant, intraspecific competition will always be strong. Selection under these conditions (*r*) favours species that produce small litters of highly competitive young. Additionally one might expect that individual longevity is increased in non-seasonal environments along with other factors which serve to heighten survivorship such as deferment of sexual maturity. Thus *r* selection represents selection for a rapid energetic turnover

and *K* selection represents selection for efficiency of exploitation (Pianka, *Am. Nat.*, **104**, 592; 1970).

In general the concept is important for ecologists concerned with the artificial control of rodent populations. Microtines from the temperate regions are typified by high reproductive output (5 litters per year; 5-8 young per litter), low survival (2-4 months) and high population densities (up to 1,000 per hectare). Heteromyids from the arid regions of the New World have low reproductive outputs (2-3 litters per year; about 2.5 young per litter), high survival (up to 5 years in the field) and low population densities (about 0.5-5.0 per hectare). At this level the concept seems to hold up well. What demographic strategies are adopted by two closely related species of rodent which occupy seasonal and nonseasonal environments respectively within the same biogeographical region? A recent study by Fleming may help to answer this question (*Ecology*, **55**, 493; 1974).

Two study sites in Costa Rica, one a dry, seasonal tropical forest, the other a wet nonseasonal tropical forest were chosen. They were separated by about 130 km. The terms seasonal and non-seasonal were used after sufficient vegetational and climatic studies had shown there to be a distinct presence and absence respectively of an annual boost in primary productivity. Fleming chose to study the population dynamics of two closely related species of heteromyid rodents, *Heteromys desmarestianus* and *Liomys salvini*. In Costa Rica *Liomys* occurs in the seasonal dry forests and *Heteromys* in the nonseasonal wet forests. Although Fleming admits that a single comparison between two species can, at best, give only weak support to a general concept, the paucity of field observations demands that a second look is taken at what data there are. *Liomys* matures earlier than *Heteromys* (3-4 months as against 8 months) and has slightly larger litters (3.8 as against 3.1). Juvenile survival is higher and mean longevity slightly greater in *Heteromys*. *Liomys* juveniles and adults weigh significantly less than comparably aged *Heteromys*.

The field study lasted only 1 year with an additional month's study almost a year later. These observations must therefore be treated with caution. The effects of other ecological factors, such as predation pressure, while their existence was noted, were not measured. Nevertheless it seems that Fleming has provided a *prima facie* field example in support of the concept made all the better by it involving two closely related species (normally regarded as *K*-selected species) in adjacent, but ecologically distinct, habitats.

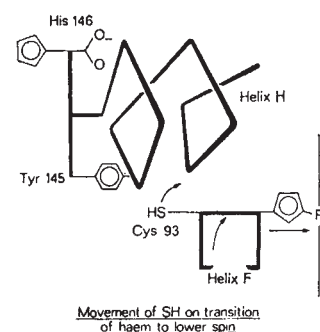
R to T with IHP

from a Correspondent

FROM a large number of interesting papers on structural and functional aspects of haemoglobin, a series of three long reports on the "Influence of Globin Structure on the State of the Heme" (*Biochemistry*, **13**, 2163, 2174, 2187; 1974) commends itself for attention. The series is noteworthy in carrying the names of ten authors from six different laboratories in the United Kingdom, the United States and Nigeria, and makes simultaneous use of a wide range of techniques.

In part 1 on human deoxy-Hb, Perutz, Ladner, Simon and Ho have used two modified haemoglobins and a mutant, all of which can be made to remain in the R (oxy) quaternary state when fully deoxygenated, and switch to the T (deoxy) state by addition of inositol hexaphosphate (IHP). By using such haemoglobin species, the effect of the R → T transition on the 5-coordinated ferrous haems can be studied in the absence of any dissociation of ligands. In the fully deoxygenated but R state, des-Arg(β-141)-Hb, NES-des-Arg(α-141)-Hb and Hb-Kempsey (β-99 Asp → Asn) all have electronic absorption spectra, ultra-violet circular dichroism and paramagnetically shifted proton NMR (nuclear magnetic resonance) spectra which differ markedly from those of normal deoxy Hb-A and resemble those of summed free deoxy α and β subunits. Addition of IHP switches them to the T state with electronic and NMR spectra like those of deoxy Hb-A. The switch also gives rise to small absorption and CD spectral changes that are most probably due to structural changes at the α₁β₂ contact area affecting the trp (β-137) and tyr (α-142) residues.

Part 2, by Perutz, Fersht, Simon and Roberts, on allosteric transitions in methaemoglobin, makes use of optical, kinetic and NMR procedures to demonstrate that binding of IHP shifts the



Effect of changes in spin state of the haem on the F helix and on the freedom of the sulphhydryl group of cysteine-93 to react with ligands. Quaternary R structure (from *Biochemistry*, **13**, 2182; 1974).

allosteric, equilibrium of high-spin methaemoglobin derivatives (aquo-, fluoro-) in favour of the T (deoxy) state. The reduced reactivity of the β -93 sulphhydryl groups is also comparable with that observed for deoxy as compared with oxy-haemoglobin. IHP seems to bind to the same site in met- as in deoxy-haemoglobin, between the terminal amino groups of the β chains. Smaller changes in the UV spectra and SH group reactivities are induced in low-spin methaemoglobin derivatives (azido-, cyano-) by IHP without the sign reversal in the CD spectrum at 287 nm associated with the R $\rightarrow$ T transition of deoxy-haemoglobin. Similarly, the NMR studies show that for the high-spin derivatives IHP causes larger changes in the ring-current-shifted resonances of some aromatic residues than it does for the low-spin derivatives. Taken as a whole, these observations indicate that solutions of high-spin methaemoglobin derivatives contain both R and T forms in equilibrium, with the R form favoured by high pH, and the T form by low pH and the binding of organic phosphates. This scheme can account qualitatively for the pH dependence of the redox equilibrium of haemoglobin and its sensitivity to chemical modification, and there is an interesting interpretation of the redox data in allosteric terms.

Part 3, by Perutz, Heidner, Ladner, Beutlestone and Slade, considers changes in the visible and near-IR absorption spectra, paramagnetic susceptibility, paramagnetically-shifted proton-NMR and electron spin resonances induced in high- and low-spin methaemoglobin derivatives by IHP, and relates them ultimately to changes in the distances between the iron atom and its ligands. The increased Fe-N bond distances observed in the T structure implies that the globin exercises tension on the haem which pulls the iron atoms further from the porphyrin ring plane, opposing the transition to the low-spin state needed for combination with oxygen and hence reducing the oxygen affinity. Finally, since haem-haem interaction is observed only when a change in quaternary structure accompanies reaction with ligands, the present findings imply that it is coupled to a change in tension at the haem, transmitted by a change in quaternary structure of the globin. This brings the discussion back to globin, which is the starting point of the series. It should perhaps be added that comprehension of the large amount of diverse experimental data is greatly helped by some summary tables and figures *en route* which summarise particular conclusions and implications reached during the course of the tightly argued discussion.

Mitotic spindle assembly *in vitro*

from a Correspondent

Two main approaches have been taken in studies of the mitotic spindle. Measurements of the birefringence of the spindle in intact cells by Inoue and others have suggested that the fibrous components of the spindle are in dynamic equilibrium with subunits. The birefringence of the spindle is lost when living cells are exposed to cold, high hydrostatic pressures and to colchicine and other antimitotic alkaloids. These agents are thought to depolymerise reversibly the microtubules of the spindle fibres and so prevent chromosome movement in intact cells. The other approach has been to isolate the intact mitotic apparatus, usually in the presence of stabilising agents such as glycols. This procedure has provided useful information about the chemistry of the mitotic apparatus but not how it works, because reversible assembly and disassembly have not been achieved with spindles so prepared.

Three groups of investigators have now reported the preparation of mitotic spindles which can be made to increase or decrease in size by appropriate treatment of the subcellular materials. The spindles are isolated in the presence of added rat brain tubulin and agents which chelate calcium, thereby preventing the disassembly of spindle microtubules. Cande, Snyder, Smith, Summers and McIntosh (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 1559-1563; 1974) lysed rat kangaroo cells with the detergent Triton X-100 into a solution of polymerisable rat brain tubulin and obtained spindles which lose and gain birefringence when cooled and warmed; the spindles can move anaphase chromosomes to the opposite ends of cell preparations. Early anaphase cells lysed into buffers containing polyethylene glycol and nucleotide triphosphates showed spindle elongation and chromosome movement in the absence of added tubulin subunits. The authors conclude that although spindle growth requires microtubule polymerisation, anaphase motions do not.

Rebhun, Rosenbaum, Lefebvre and Smith (*Nature*, **249**, 113-115; 1974) report that spindles isolated from eggs of the surf clam (*Spisula*) cooled and then warmed with chick brain tubulin showed increased birefringence in the form of fibres similar in distribution to the mitotic apparatus of living cells. Sometimes such artificially polymerised spindles were larger than normal spindles. The isolated spindles increased in length but did not shorten and were stable to dilution of tubulin. It seems that they can assemble tubulin more

readily than they can break down the assembled units.

Inoue, Borisov and Kiehart (*J. Cell Biol.*, **62**, 175-184; 1974) found that tubulin purified from porcine brain augmented the birefringence of *Chaetopterus* spindles. The cells were lysed in a hypotonic calcium-chelating solution, and in the presence of tubulin, spindles and asters grew considerably larger than those in intact cells. The isolated augmented spindles depolymerised rapidly at 6°C, and birefringence was slowly recovered upon return to 23°C. The birefringence of the spindles also decreased in the presence of 2.5 mM calcium but not on dilution of tubulin or in the presence of colchicine or colcemid.

The results represent a useful advance in knowledge of the mitotic apparatus. They show that the conditions required for polymerisation of isolated spindle microtubules are similar to those defined by Weisenberg and others for tubulin from various sources, including surf clam eggs. The response of the isolated spindles to temperature reflect those observed in intact cells, but other responses, such as those to colchicine do not. Disassembly does not follow dilution of tubulins, so that a simple equilibrium between the protein subunits and spindle microtubules is unlikely to explain fully the behaviour of the spindle. Perhaps improvements in isolation technique will produce spindles that perform more like those in intact cells. Since exogenous enzymes, cofactors and inhibitors can gain access to the isolated spindles, it should be possible to analyse their role in spindle formation and disassembly. The spindle, however, is itself likely to contain a complex mixture of enzymes and other constituents, so that the analysis will not be straightforward.

Gibbons and others have found how useful sperm tail preparations made accessible to exogenous agents by detergents can be in analysing flagellar movement, and similar analyses should be possible with isolated spindles. It would be of great interest to know whether spindle assembly or disassembly depends on reversible polymerisation of tubulin and whether the presence of dynein, actin or some other contractile protein is required for chromosome movement. Apparently low calcium is necessary for assembly, as well as phosphorylation of GDP tightly bound to tubulin. Jacobs at King's College London, has recently been studying a transphosphorylase associated with tubulin, but separable from it, which seems to be involved in the assembly process. Among the many possible systems involved in the control of spindle assembly and action are the concentration of calcium and level of transphosphorylase activity.

The commonplace and unexpected from high energy physicists

from David J. Miller

LAST year's wonder has become this year's commonplace. On July 1, the opening day of the International Conference on High Energy Physics at Imperial College, London, five separate new pieces of evidence for neutral weak currents were reported. Moreover, the two groups who had published previously reported greatly improved statistics (see *Nature*, **245**, 119; 1973 and **249**, 211; 1974 for discussions of the first neutral-current result, from the Gargamelle bubble chamber at CERN, Geneva and of the early Harvard/Fermi Laboratory results).

The most striking of the new pieces of data comes from a group at the California Institute of Technology, which used an array of counters, spark chambers and magnets in a neutrino beam at the Fermi National Accelerator Laboratory (FNAL). The momentum of their neutrino beam is known better than in the first Harvard-FNAL experiment, and they have a clearer technique for identifying events without fast muons. Their ratio for muonless neutrino events (neutral-current mode) to events with a muon (charged-current mode) is 0.22 to 1. With an antineutrino beam the equivalent ratio is 0.33 to 1. Although their neutrino beam momenta are around 45 GeV/c or 120 GeV/c, compared with around 1 to 10 GeV/c in Gargamelle, this neutrino ratio is very close to the latest Gargamelle figure, and the antineutrino ratio is not alarmingly different, considering the greater uncertainties involved in small antineutrino statistics. A collaboration of 'East Coast' university groups, working at the 29 GeV/c Brookhaven accelerator near New York, has also seen a clear neutral-current signal in a spark chamber experiment.

Two groups reported the observation of neutral-current events with all the final-state particles identified. In Gargamelle and in the big spark chamber arrays, this has been difficult, since the neutrinos interact with the protons or neutrons of a heavy nucleus. At the Argonne Laboratory, near Chicago, a group from Argonne, Concordia College and Purdue University has used the 12-foot bubble chamber, filled with liquid hydrogen or deuterium, to identify about 14 events in which a neutrino struck a single proton (or a neutron in deuterium). The final state in each of these events contained a positive or a neutral pion, plus a recoil neutron or proton (and also a visible 'spectator' proton, in deuterium). As

with all neutral-current experiments, the final-state neutrino in each event was not observed. The numbers are too small, to date, to do more than to establish the existence of these processes. Experimenters from CERN obtained similar results by studying neutrino interaction on the free protons in propane, using film which was taken 7 or 8 years ago, before the existence of neutral currents was even suggested. It demonstrates that there should be a great deal of useful data to be obtained from the next Gargamelle run, with a propane filling and a more intense neutrino beam.

There can now be no doubt that neutrinos and antineutrinos interact with nucleons (that is, protons and neutrons) in two distinct ways. The charged-current mode is more copious, but the neutral-current mode, in which another neutrino goes off afterwards rather than a charged muon, is well established. It is even becoming possible to put realistic limits on the 'Weinberg angle', a parameter which governs the relative strength of the charged and neutral currents in the simplest unified theory of weak and electromagnetic interactions.

One interesting question still awaits a definite answer; that is, how do neutrinos and antineutrinos interact with electrons? The Gargamelle collaboration reported the observation of two events in which an electron appears to have been produced by an antineutrino interaction. Although the calculated background is small, there can be no certainty in the observation of such a small signal. But the rate is roughly what would be expected within the framework of the simplest theory, using a value for the Weinberg angle which is consistent with the neutrino-nucleon experiments. This is the first experimental evidence that the neutral neutrino current might interact with electrons as well as with protons.

Muon puzzles

There was speculation at the meeting about both new elementary particles and new interactions. One new effect is the direct production of charged leptons from hadron collisions. The leptons—electron, muon and neutrino—have never been observed to take part in strong interactions. The hadrons—the proton and neutron, the hyperons, the mesons and all of the resonant states—are the only known strongly interacting particles. They were thought to produce

leptons only in weak or electromagnetic decays, not in their strong collisions.

A group from CERN, Columbia and Rockefeller Universities in New York, and from Saclay in France, working at the CERN Intersecting Storage Rings in Geneva, has now found convincing evidence that electrons are directly produced in proton-proton collisions at the very highest observed energies. Another group from Columbia, working at the Fermi National Laboratory near Chicago, has seen the same sort of effect, with both electrons and muons coming from proton-proton collisions at somewhat lower energies. A Chicago, Harvard, Pennsylvania and Wisconsin group also has seen direct muon production at the Fermi Laboratory, and direct muon production has been reported from the even lower energy accelerators at Serpukhov in the Soviet Union and at Brookhaven, New York. All of these data have, of course, been corrected for indirect muon production by means of the weak decays of mesons. In every case the ratio of direct lepton production to pion production is about 1 to 10,000. The ratio does not vary much over the large energy span of these different machines. It also seems roughly constant as the transverse component of the lepton momentum, perpendicular to the incoming protons, is varied from 2 to 5 GeV/c.

Two sorts of explanation have been suggested for direct lepton production; the revolutionary or the merely surprising. One revolutionary explanation requires that 'charmed' particles are being produced, and decaying rapidly to normal particles including muons or electrons. 'Charm' is a postulated new quantum number, similar to the old-established 'strangeness' quantum number. It is needed to explain, among other things, the absence of strange-particle production in neutral-current weak interactions. The merely surprising explanation requires that most of the pion production in high energy proton-proton collisions actually comes from the production of massive resonances which decay to pions by the strong interaction. Some of these resonances, in particular the 'rho', the 'omega' and the 'phi' known as the 'vector mesons', also decay occasionally by means of the electromagnetic interaction to a pair of electrons or a pair of muons. Preliminary calculations, using vector meson decays, give a lepton to pion ratio of about 1 to 100,000. Nobody knows yet whether the extra factor of 10 can be found to make this calculation match the observed rate. But even if the factor can be found, and a revolutionary explanation is not needed, ideas of particular production at high energies will have been radically changed.

The Fermi Laboratory and Harvard

neutrino experiment has revealed two puzzling muon events of quite a different kind. Each seems to be the result of the interaction of a 150-GeV neutrino in their apparatus, producing two muons and a shower of hadrons. Ordinary 'charged-current' neutrino interactions contain one final-state muon, and neutral-current events have no muons. As a theorist remarked at the conference: "taking preliminary results seriously is a well known way of making a fool of yourself". Nevertheless, there is much speculation that these dimuons could be due to the decay of a totally new kind of particle. Some say 'charmed' particles again; some say it may be the 'intermediate boson' that carries the weak force, a sort of heavy photon; some say it is a heavy lepton of the kind needed in the unified weak and electromagnetic theory of Georgi and Glashow. Certainly, if this observation is correct, then no 'merely surprising' explanation will do.

Prospecting for a dead slab

from Peter J. Smith
Geomagnetism Correspondent

LITHOSPHERIC slabs descending beneath trench systems are known to have seismic velocities which are measurably higher than those in the surrounding mantle. Because such downthrusting slabs can be recognised in simpler and more convenient ways, not least by their deep seismicity, there may be little need to use the seismic velocity property to detect Benioff zones which are still active. But what about zones in which subduction has now ceased and which are seismically quiet or dead? When subduction ceases, the relevant slab, initially cooler than the mantle into which it was previously descending, will slowly warm until the thermal and compositional contrast between it and the surrounding mantle disappears; but until this process is complete the 'dead' slab may retain enough of its identity to maintain the velocity contrast. So can this contrast be used to detect the slab's presence?

That there are likely to be recently dead slabs to detect may be inferred by extrapolating known plate tectonic processes backward in time. The evolution on the western margin of North America is a good case in point and probably the most-studied example. Magnetic anomalies in the north-east Pacific suggest that a Farallon plate (sometimes called the Juan de Fuca plate or the Gorda plate) descended along the boundary between it and the North American plate until about 30 million years ago, at which time a spreading ridge between the Farallon and Pacific plates began to collide with

the western North America trench and strike-slip motion commenced along the San Andreas fault. In this way it is thought, the plate boundary along western North America began to change from a subduction zone to a transform fault. There is evidence from seismic reflection studies of the continental margin, from the occurrence of subcrustal earthquakes, from andesitic volcanism in the Cascades and from marine magnetic anomalies that north of the Mendocino fracture zone subduction may still be occurring very slowly. South of Cape Mendocino, however, subduction has ceased, although Atwater (*Bull. Geol. Soc. Am.*, **81**, 3513; 1970) has concluded that the cessation may have occurred no more than a few million years ago.

The implication here is that the remains of the Farallon slab may still lie beneath California and may still have sufficient of its original identity to produce a measurable velocity contrast. To test whether this is indeed so, Solomon and Butler (*Earth planet. Sci. Lett.*, **21**, 421; 1974) have attempted to detect such a contrast by analysing the travel-time delays of teleseismic P waves in the region. The method adopted was derived from that used by Davies and McKenzie (*Geophys. J.*, **18**, 51; 1969) to show that shallow earthquakes and explosions above a descending slab produce distinctive patterns of travel-time residuals for teleseismic P waves as a result of the velocity anomaly within the slab. The basic residuals obtained by Solomon and Butler were the travel-time delays at each station concerned for P waves from many different earthquakes and explosions. But to remove uncertainties in source locations and origin times and to reduce near-source contributions to the total travel-time residuals, the residuals actually plotted were the differences between the travel-time delays at each station and the corresponding delays at a reference station overlying what is presumed to be more uniform mantle. The sources were mid-plate earthquakes and explosions, deep earthquakes, and "carefully screened" (to avoid obvious near-source heterogeneities) earthquakes on spreading centres and transform faults.

In the absence of any detailed knowledge of the position and extent of the supposed dead slab, the stations chosen were those between latitudes 37°N and 50°N and between longitudes 116°W and 125°W at which P wave arrivals had been regularly reported during the period 1964–1970 (a selection process which eliminated all but seven stations, three of which were regarded as reference stations). The residuals obtained from various station pairs were plotted on residual-spheres, the resulting plots being a measure of the variation of travel-time delay with direction of wave

propagation in the upper mantle beneath the relevant station.

The residual-sphere for station LON in the Cascades in Washington (referred to BMO, Blue Mountain Observatory to the east) failed to indicate any systematic trends in residuals. If a high velocity slab lies in the upper mantle beneath western Washington and if its upward projection cut the Earth's surface near LON, an eastward-dipping band of negative residuals (early arrivals) might be expected. No such band was observed. But for station pairs based on the three primary stations in California (MIN in the southern Cascades, ORV and JAS along the western edges of the Sierra Nevada), a group of consistently negative residuals was observed towards the east in each case. Moreover, the average travel-time advance in the eastern quadrants of the residual-spheres (calculated in the same way for each sphere) decreased southwards from 1.2 s for MIN, through 0.8 s for ORV, to 0.5 s for JAS.

These patterns of travel-time residuals (irrespective of magnitude) are consistent with the presence of a dead slab dipping eastwards beneath California. On the other hand, they cannot be said to prove the point absolutely because the same data could in principle be explained using a model with undulations in the depth to the top or bottom of the low velocity zone. Moreover, the data are not as complete as they might be because of a paucity of seismic sources in eastern North America. But seen in the light of other evidence the case for a dead slab is more convincing. In addition to the plate tectonic inferences already quoted, support may be adduced from heat flow studies, for example. According to Roy *et al.* (in *The Nature of the Solid Earth*, McGraw-Hill, 1972), the low heat flow in the Sierra Nevada apparently requires a heat sink in the shallow (~50 km) mantle—a role that could easily be filled by the postulated slab. If this interpretation is accepted, the implication then is that between the Pacific plate and the Sierra Nevada the Farallon plate dips at an angle of perhaps no more than 10°–15°. A more detailed analysis of the travel-time data, on the other hand, suggests that east of the Sierra Nevada the dip is much more steep (possibly 40°–50°).

The southward decrease in the magnitude on the travel-time advance could result from differences in the positions of the stations with respect to the position on the Earth's surface of the upward projection of the supposed slab, or it could be due to a real southward decrease of the P wave velocity in the slab. Solomon and Butler favour the latter explanation on the grounds that the three stations are similarly

located very close to the western margin of the Sierra Nevada or the California Cascades, but admit that the evidence is not sufficient to rule out the other possibility. On the other hand, from Atwater's models, they deduce that subduction ceased beneath station MIN about 4 Myr ago, beneath ORV about 6 Myr ago and beneath JAS about 12 Myr ago. In other words, the dead slab seems to age towards the south.

This theory would offer a natural explanation for a southward decrease in P wave velocity, for the older the slab the more it will be thermally assimilated with the surrounding mantle and thus the lower will be the mantle-slab velocity contrast.

Eyeglow in butterflies

from our *Insect Physiology Correspondent*

It is a familiar observation that the dark-adapted eyes of nocturnal moths produce a conspicuous glow when viewed in a beam of light. The 'tapetum' responsible for this reflection is believed to be the rich network of air-filled tracheoles which invest the deepest levels of the retina. As Horridge (*Proc. R. Soc. Lond.*, **B179**, 98; 1973) pointed out, if the light entering one facet is confined to a single rhabdom (as in the light-adapted eye) its reflection will not emerge through another facet. On the other hand, whatever the precise mechanism of eyeshine may be, in the dark-adapted eye the scattered light may follow many possible routes within the eye and emerge through other facets.

Particular interest has been taken in the eye glow of butterflies, which can be very brilliant, and of diverse colours, in the dark-adapted state; but which disappears very rapidly on illumination. Miller and Bernard (1968) attributed this light to the flange-like taenidia of the tracheoles running vertically between the bases of the rhabdoms, which they suggested were functioning as a quarter-wave stack made up of alternating layers of air and cuticle with refractive indexes of 1.0 and 1.4 respectively. Swihart *et al.* (*J. insect Physiol.*, **20**, 359; 1974) now question this 'basal reflection theory' for the origin of eye glow in butterflies.

These authors concentrated on butterflies of the genus *Vanessa*, which show a uniform orange reflection over almost the entire eye. The band of wavelengths reflected is far narrower than was to be expected from a quarter-wave stack. The time required for the extinction of the eye glow is constant at some 5–10 s for a given eye; the cycle of extinction (and reappearance in the dark) can be repeated hundreds of times with little

variation. No detectable changes in pigment distribution can be detected. As Exner had shown, the response can be completely localised to a few ommatidia. The reflections are more intense than would be expected from a basal reflection—perhaps 20 to 50% of the incident light—and they can be upset by the slightest mechanical contact with the cornea.

Furthermore, the authors show that during light adaptation there is a photo-mechanical contraction of two specialised retinula cells; this causes the rhabdom to fold and the crystalline cone to be withdrawn away from the cornea. The elastic 'corneal process' between the cornea and the crystalline cone is stretched and its optical properties are altered. The corneal process is believed to be the source of the eyeglow reflections, the spectral properties of which will be largely determined by the overlying cornea. When the corneal process is stretched the reflections are extinguished. This 'distal reflection theory', according to the authors, has no physiological implications for vision in butterflies: these are diurnal creatures; at the levels of illumination they normally encounter the eyes are in the light-adapted state and the corneal process does not reflect—it is simply a portion of the dioptric apparatus, serving to conduct light from the cornea to the lens.

How swamp plants react to thermal pollution

from Peter D. Moore
Plant Ecology Correspondent

THERMAL pollution is a problem of the future; as nuclear reactors proliferate, so larger quantities of waste heat will be pumped into the environment causing problems of thermal tolerance to the organisms living in their vicinity. To date little is known about the effects on wetland plant communities of artificially raising the temperature, although some information exists regarding aquatic organisms.

Sharitz (*Oikos*, **25**, 7; 1974) has been able to study the effects of reactor effluent in a situation where adequate controls have been possible—in the swamp forests of the south-eastern United States. Many of the lower stretches of rivers in this area are surrounded by swamp forests which, as the term implies, are dominated by trees, such as *Acer rubrum*, *Fraxinus pensylvanica*, *F. caroliniana* and *Planera aquatica*. In these swamps, 48% of the plant species are trees and a further 14% are shrubs and woody vines. Swamp forests experience considerable

seasonal fluctuation in water table and most of the tree species are intolerant of prolonged flooding. Even *Taxodium distichum* and *Nyssa aquatica*, which are the most tolerant to floods, are killed by lengthy exposure to a high water table.

Part of the Savannah River catchment in South Carolina is occupied by a nuclear reactor of the US Atomic Energy Commission, which pumps hot water into some of the tributary streams. One stream in the area is unaffected (providing a convenient control), two others are thermally polluted and a fourth has been recovering for more than 4 years following 14 years of thermal stress. The area therefore provides a useful setting for the study of the effect of nuclear plant discharge on swamp vegetation and the way in which the flora recovers after the activity is stopped.

The area currently polluted has water temperatures which frequently exceed 45°C and the vegetation of the area has been profoundly altered. All trees, shrubs and lianes have been destroyed and the only habitats suitable for plant growth are the islands formed from collapsed, dead timber; here some herbaceous plants, such as *Ludwigia leptocarpa* have become established. In fact the diversity (Shannon-Weaver index) of the ground flora is fractionally increased, resulting from the new microhabitats created.

The recovery area has similar stumps and logs, together with emersed, flood-plain sediments which have been colonised by adventive herbs, such as *Polygonum punctatum*; a large proportion (35%) of the plants of this area are annuals. Some woody plants are recorded, including *Pinus taeda* and *Salix nigra*, but these are not species typical of the mature swamp forest, so that it is difficult to judge how long the area will take to return to the typical swamp forest if, indeed, it ever does.

The loss of woody species during flooding could be of particular concern if it resulted in local erosion and consequent silting of the Savannah River. It is unfortunate, however, that it has not been possible to distinguish between the truly thermal effects of disturbance and those resulting from a permanent raising of the water table. It is probable that the loss of trees and shrubs is due to flood intolerance rather than sensitivity to high water temperatures. Such a distinction, were it possible, would indicate whether the destruction of the swamp forest could be avoided by providing drainage conduits direct to the Savannah River. Although such action would stabilise the forest, it would not, of course, reduce the effects of thermal pollution on aquatic organisms in the river.

Photochemical war on the atmosphere

John Hampson

Centre de Recherches sur les Atomes et les Molecules, Faculté des Sciences, Université Laval, Quebec, Canada

Professor Hampson believes that many scientists have not recognised the potential danger of thermonuclear warfare. He outlines some of the changes in the photochemical regime of the atmosphere, which may be wrought by nuclear explosions, and suggests that these effects must be considered in future talks on arms limitation.

A FIVE per cent increase in the atmospheric content of ozone in recent years has been attributed to the recovery of the atmosphere following the injection of NO_x by nuclear tests during the winter of 1961–62¹. Estimates of the theoretical quantity of NO_x generated have been presented by several investigators^{2,3}. If the NO_x is not swept out of the stratosphere by the change of heating and transport induced by the new ozone structure, then, should the amount of nuclear discharge into the atmosphere increase by one order of magnitude, the total ozone content may be halved. At first sight it seems that the variation should follow a square root law. If the decreased screening by ozone, and the consequent increase in NO production is allowed for, however, then for particular values of the reaction rate coefficients and circulation parameters a linear dependence is a closer estimate of the maximum possible effect (within the possible range of errors). A further increase of one order of magnitude could reduce the total ozone in the atmosphere to a fraction of its present value. It is appropriate to enquire into the effect of such radical changes in the ozone content and the circumstances in which they might occur.

The photochemical behaviour of the stratosphere is far from fully understood, and the chemical effects of high yield, high altitude nuclear explosions have never been measured and reported. It is probable that there are changes besides those which are predicted by the simple NO_x theory. That is, however, no reason to reject the theory, for it forms a solid base for future work. It is difficult to believe that mankind can put into the chemosphere an energy comparable to the chemical energy of the ozone layer (~3,000 Mton) in less time than it took to create the layer, and still expect the chemosphere to remain intact.

It has been assumed⁴, and no alternative view has been presented, that the evolution of life on the exposed surface of the planet began with the formation of the ozone screen. The removal of this screen would mean that conditions of ultraviolet illumination at the surface would become similar to those of Precambrian days, and would presumably eliminate all surface life. The biosphere involves such a complex pattern of interacting influences that it is impossible to assess the effects of drastic reduction in ozone. 'Reasonable' approximations must be used. The ozone content in the path from the Sun to the summer pole is double that in the shortest Sun-surface path at the equator. Halving the ozone abundance would create a situation where the ultraviolet input is everywhere greater than it was previously. For want of a better yardstick I shall assume that this is the degree of ozone reduction which

could destroy the present biosphere. The argument here is simply that the biosphere has adapted to the available ultraviolet illumination, and a critical condition exists. Following a halving of the ozone content no living organism would be able to find a place on the surface of the Earth where the ultraviolet input was equal to or less than that previously available anywhere.

It is instructive to consider the circumstances in which a thermonuclear war could occur, halving the effectiveness of the ozone screen. The structure, deployment and potential use of nuclear weapons are dictated by an analysis of the probable damage and potential injury which would be inflicted on the 'opponent'. It seems reasonable to assume that it will occur whenever the predicted outcome would be more favourable to the instigator than any alternative action at his disposal. There is no evidence that any of the nuclear powers, or any of the nations who are allegedly attempting to forestall nuclear proliferation through the United Nations, have made any attempt to consider the photochemical problem in their deliberations. It must therefore be assumed that the decisions on thermonuclear war are based on a hypothetical body count of potential victims. That is the way the issue is posed in the published studies of nuclear war, particularly exemplified in the account by Kahn⁵.

To knock out one of the major powers, the United States, the USSR or China, 5,000 Mton must be accurately delivered, somewhat less would be required to destroy Europe. If the concepts and calculations of Johnston¹ are correct the use of the minimal deterrent would halve the ozone content. In these circumstances, the deterrent as such is a myth and becomes one of Kahn's Doomsday Machines⁶.

If NO_x is the critical reactant which is destroying the ozone then the effects of thermonuclear weapons may be greater than the estimates I have given. These estimates are based on low level bursts in which the NO_x concentration is calculated from consideration of the thermodynamic equilibrium in the fireball at a temperature of 2,000 K. A high proportion of the nitrogen atoms produced before and during that stage, during the development of the fireball, are recombined as N_2 , and the NO_x production represents a small proportion of the total energy of the nuclear burst. This is not the case for an explosion at high altitude, when the energy density of radiation below the burst point, at the altitude of maximum NO production, is sufficiently small that recombination through $\text{NO} + \text{N} \rightarrow \text{N}_2 + \text{O}$ plays a proportionally smaller role. If the concentration of N is small compared with that of O_2 , then the bulk of the NO produced by $\text{N} + \text{O}_2 \rightarrow \text{NO} + \text{O}$ may not be removed subsequently in the competing reaction between N and NO. High altitude explosions are much more efficient at producing NO than are ground level explosions. It is fortunate that the tests in 1962–63 were conducted in the lower atmosphere and not at high altitudes. Otherwise there may have been such a large production of NO that any subsequent debate on atmospheric chemistry would have been both impossible and superfluous.

Each of the two major nuclear powers seems to have

a stockpile in excess of the critical value, 5,000 Mton, and to be continually jockeying for a uniquely unassailable position. The only way this can be achieved is by using ballistic missile defence, and the nations concerned can be expected to make this a priority item of defence research. That constitutes a somewhat greater danger to the ozone screen than do the offensive weapons. It not only increases the required number of offensive weapons but also creates more NO per unit of energy released into the atmosphere, because interceptions must be made at high altitudes. Some years ago there was a far fetched, fanciful concept that the release of 1,000 Mton or so in the equatorial E region might have produced an artificial Van Allen belt of sufficient intensity and duration to clean up a great deal of the debris and decoys which clutter and confuse the radar return from a missile warhead. It would not have worked, but it is disturbing to discover that such concepts are advanced with absolutely no consideration of the potential effects on the Earth's photochemical system. Even the 'orthodox' defence systems agreed to in the Strategic Arms Limitation Talks (SALT) are themselves a major threat to the photochemical environment. Several hundred interceptors are involved, each with warheads in the Mton range. The actual yields of the interceptor warheads have not been published. Assuming that they may now, or eventually, be set at 10 Mton, which would give the maximum capacity for damaging the re-entry warhead and cleaning up the accompanying assorted objects, then the defence systems defined by SALT, which themselves give only a token and limited defence, would be capable of injecting 2,000 Mton into the atmosphere. They would do this at high altitudes and therefore have a much greater potential for modifying the NO_x environment than the low altitude bursts.

The threat to the ozone layer posed by thermonuclear discharges is much more serious than that posed by contamination from supersonic transport (SST) flights. We have time to analyse the photochemical environment and assess the potential effects long before widespread SST flights add an appreciable amount of NO_x to the environment. The bigger issue is whether the nuclear deterrent is a Doomsday Machine. It is astonishing that none of the deliberations on reduction of nuclear arms pay the slightest attention to this photochemical parameter. Surely there is a need to establish whether there is an absolute limit to nuclear war, involving energies considerably smaller than those at present used as a deterrent. Such information could be used as a lever to cajole the nuclear powers to move towards a more positive view of the need for arms control. Johnston¹ has performed a considerable service in drawing attention to the potential fragility of the ozone screen. It is to be hoped that increased attention will be paid to the effects of thermonuclear weapons and that this may provide a rationale for the reduction and control of nuclear armaments.

A *prima facie* case for NO as the agent controlling ozone having been clearly established, it should be noted that there are many factors of the chemistry and circulation of the ozone layer, and of the interrelation between the layer and the rest of the Earth-Sun system which have to be researched before the case is proven. This should be no excuse for disregarding the potential danger. It does, however, imply that we may find that nuclear weapons could exert dangerous effects in other, more subtle, ways.

A single, 10 Mton weapon exploded in space, less than an Earth's radius away, throws out about as much energy at the Earth as does the solar wind during the passage of the interplanetary magnetic field sector boundary. Can it be assumed that this could cause effects commensurate with those deduced by Roberts *et al.*⁶? If a 10% change in vorticity could occur with a single weapon, what would a space war do? We need to examine whether this effect

stems from a chemical source (see ref. 6).

There seems to be a disparity between the hydrogen escape deduced from observations at the exobase, and models of H transfer from the lower atmosphere to the exobase. In view of this can we neglect the possibility that a nuclear war in space would lower the hydrogen concentration above 40 km, and through an increase in the upper atmospheric ozone content reduce the formation of NO and increase the total ozone content?

We simply do not know the answers to many important questions. Granted an adequate programme of measurements, far more ambitious than that envisaged today, and sufficient clairvoyance (omniscience might be a better term) the minimum time necessary to assess natural variations resulting from solar activity in the chemosphere will be one solar magnetic cycle of 20 years. There can be little prospect of accurately defining how contamination can compare with or can couple into the natural variations in a shorter period.

We might as well accept both of the apparently contradictory propositions that contamination by NO_x can change the ozone abundance and that the observed increase in ozone is real but is not related to changes in the NO_x content.

From Johnston's estimates¹ it seems reasonable to infer that a fleet of 100 Concorde might change the ozone content over a period of 5 years to an extent comparable with the observed rate of increase of ozone during the decade 1960-70. This fleet seems to correspond to the maximum scale of production and deployment to be expected between now and 1980. There seems to be adequate time between now and 1980 to refine the observations and theoretical analyses of the chemosphere to the point at which we can estimate a 'limiting, scale of operations for supersonic aircraft and other potential sources of stratospheric contaminants. This period of grace should be used to ascertain whether the observed increase in ozone during 1960-70 continues into the next decade and is therefore not related to recovery from the nuclear tests.

Contamination by CO₂ is recognised, but can hardly be averted at the moment. Studies of the effect of changes in the CO₂ content have been limited to evaluations of the greenhouse effect in the lower troposphere. No attention has been paid to possible alterations of temperature and vertical stability above the tropopause. These can modify the transport of reactive minor constituents, and readjust the photochemical balance. There is insufficient information on the details of the nitrogen cycle. We cannot be absolutely sure that the scale of human modification of the critical elements in the cycle will have no effect.

The effects of nuclear weapons are another matter. The majority of the scientific community fail to recognise the potential danger from a change in the chemical envelope of the Earth. They thus fail to consider the possibility of defining a limit to nuclear activity, which can be universally agreed upon and could set the stage for arms control. So long as the nuclear problem is viewed as one of international rivalry there can be little prospect of any control because it will always seem that one nation can gain an advantage over another. Kahn's dictum that a Doomsday Machine can never be used is a reasonable enough basis for arms control. But this depends on our being able to recognise such a machine.

The main motivation for stratospheric research should be the desire to delimit the effects of general contamination and to define the absolute limit of nuclear activity which would constitute 'The Doomsday Machine'.

The fine structure (both spatial and temporal) of the reactive minor constituents deserves careful study. Johnston *et al.*¹ and Goldsmith *et al.*³ present arguments that it is the long term global effects of nuclear tests on the ozone content, and not short term local effects, which

should be considered. This is an oversimplification. The form of polar stratospheric circulation and the characteristics of the auroral belt were abnormal during the winter of these tests. It is always possible that there have been more complex interrelationships between the tests and the photochemical regime than has been supposed.

All the parameters involved must be studied, and all potential, alternative models of stratospheric chemical behaviour must be included in order to avoid a superficial interpretation which fits the selected facts visible from the fraction of the iceberg that can be seen at present.

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Hopping losses in polarisable dielectric media

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The loss in most dielectric materials shows a component that is 'flat' in frequency over several decades and does not depend strongly on temperature. The usual interpretation as the sum of many Debye-like loss peaks is criticised and a new model is proposed in which charge carriers hop between localised sites in a polarisable medium.

THE dielectric loss $\chi''(\omega)$ is defined as the imaginary component of the complex dielectric susceptibility

$$\chi(\omega) = [\epsilon(\omega)/\epsilon_0] - 1 = \chi'(\omega) - i\chi''(\omega) \quad (1)$$

where $\epsilon(\omega)$ is the dielectric permittivity, ϵ_0 is the permittivity of free space and ω is the radian frequency. The corresponding a.c. conductivity is $\sigma'(\omega) = \epsilon_0\omega\chi''(\omega)$ and this may exceed the d.c. conductivity σ_0 by some orders of magnitude. The experimentally observed frequency dependence of dielectric loss in a wide range of materials may be expressed by the empirical relation

$$\chi''(\omega) = \Lambda(T)\omega^{n-1}$$

where $\Lambda(T)$ is weakly dependent on temperature but is not generally characterised by a simple activation energy and the exponent n lies in the range $0 < n < 1$, with typical values between 0.6 and 0.95. This implies that the loss is 'flat' in frequency, often over many decades in the normally accessible range between sub-audio and microwave frequencies¹⁻⁵. In general, there seems to be no correlation between the values of σ_0 and $\sigma'(\omega)$ for various materials.

There are two different approaches to the theoretical interpretation of the flat loss given by equation (2). The first is applicable to materials which are believed to contain molecular dipoles and it involves the assumption of a suitably wide distribution function $g(\tau)$ of dipolar relaxation times τ , covering several powers of ten⁶.

The second approach is that of the electronic hopping school which considers the frequency dependence of the localised charge carriers hopping in a random array of centres^{3-5,7,8}. This 'sequential hopping' model is in many ways equivalent to the former approach, replacing the distribution of dipolar relaxation times with a corresponding distribution of hopping times. But it also provides for the d.c. conductivity in the limit of zero frequency. Given a sufficiently wide distribution of intercentre spacings and of their relative energy differences, it is possible to obtain a frequency dependence of the type given by equation (2) and extending over a reasonably wide range of frequency. Another explanation invoking local atomic movements in disordered solids has been proposed by Pollak and Pike⁹.

Hopping model

I have expressed some doubt about the universal applicability of the sequential hopping model in view of the fact that a very wide range of different materials show a very similar dependence on frequency and the absolute values of the dielectric loss fall in a relatively narrow range². The lack of correlation between σ_0 and $\sigma'(\omega)$ is also unexpected in the hopping model, as is the apparent independence of $\sigma'(\omega)$, but not of σ_0 , on pressure^{10,11}. Furthermore, the same frequency dependence is seen in relatively thick samples in which sequential hopping represents a plausible model and in very thin (1 to 3 nm) samples, in which there is no scope for more than one hop¹².

So I believe that there is sufficient uncertainty about the origin of the flat dielectric loss due to hopping carriers to make it desirable to propose an entirely new physical model in order to evaluate its merits and consequences. I consider that an essential feature of this model must be its general applicability to a wide range of semiconducting and dielectric systems. It is relevant to recall here that it has been suggested that the flat loss in some conventional dielectric materials may also be due to electronic hopping, in view of the known presence in these materials of low mobility carriers^{13,14}. A detailed analysis of the behaviour of dipolar systems will be published elsewhere.

Hopping in a polarisable medium

In the conventional picture one considers hopping as occurring between two localised levels, each of which is uniquely defined in space and in energy (Fig. 1a). This model is applicable to media in which the polarisation responds sufficiently rapidly to the appearance of an electron on any one site so that the transition may be said to occur effectively into the final state. But if the polarisation of the dielectric medium responds relatively slowly in comparison with the time taken by the tunnelling process, as is the case almost by definition in all materials showing a strong dispersion of conductivity with frequency, then the energy level picture must be as shown in Fig. 1b. The 'empty' and the 'occupied' states are separated by a polarisation energy W_p , due to the relaxation of the surrounding lattice and carriers.

In this case, the transition from a localised level i into an equivalent unoccupied level j having the same energies may be considered to occur in three stages (Fig. 1c). Process 1 is the thermal excitation of the carrier into a virtual state corresponding to the unrelaxed energy of an empty state. The following tunnelling transition (2) is not activated and the process is completed by a gradual relaxation, 3, into the ground state of the newly occupied centre.

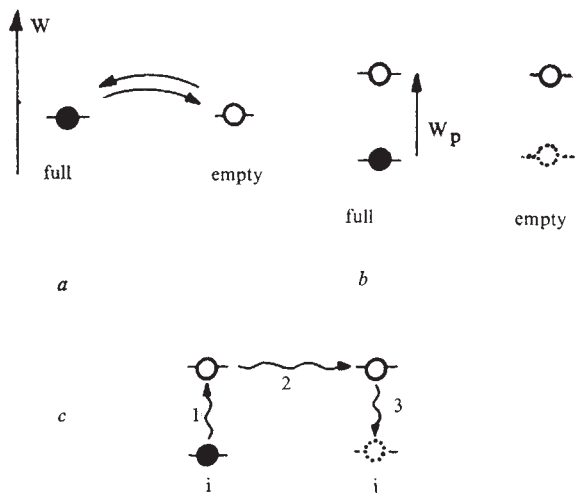


Fig. 1 Localised energy levels in hopping between pairs of centres. *a*, Hopping in a medium in which polarisation relaxes very rapidly—the conventional hopping system; *b*, full and empty centres in a medium in which polarisation relaxes slowly; *c*, three stages of hopping between two centres as in *b*.

In thermal equilibrium detailed balancing determines the transition rates $\gamma_{ij}^0 = \gamma_{ji}^0 = 1/\tau_0$ at which the gain of energy from process 3 exactly balances the energy required by process 1. For this mechanism to be applicable we require that the equilibrium transition time τ_0 should satisfy the condition

$$\tau_0 \gg \tau_p \quad (3)$$

where τ_p is the relaxation time for the polarisation process, since otherwise the carrier would be re-excited before it has had a chance to relax. This condition may be expected to be satisfied at low temperatures, such that $kT < W_p$, and at higher temperatures the system becomes effectively the same as the model of Fig. 1*a* corresponding to a rapidly polarisable medium, since in this case the model has no time to respond to the rapid transition rate and adjusts itself only to the mean occupancy.

The proposed slow polarisation mechanism is not necessarily confined to materials with polar lattices—it may also be expected in otherwise non-polar materials which contain hopping carriers responding to any changes of the spatial distribution of other carriers in their neighbourhood.

I now consider an assembly of N identical pairs of localised sites, each pair containing one electron capable of jumping between the two sites constituting the pair. I shall assume for simplicity that the sites have the same energies—this does not entail any loss of generality of the subsequent argument, while simplifying the discussion. In the absence of external fields the occupancies of the individual sites are equal on average and are $f_0 = 1/2$.

If an external field is applied to the system favouring 'downstream' transitions and discouraging 'upstream' ones, the occupancies of the corresponding centres change by $\pm f'$ resulting in a net polarisation

$$P = ea \sum_s f'_s \quad (4)$$

where a is the distance between sites and the index s denotes summation over pairs of centres.

The perturbation of the occupancies of the system by the applied field must result in a net loss of energy W_p for each transition in excess of the equilibrium rate $1/\tau_0$. We argue that at this excess transition rate the energy derived from the relaxation process 3 in Fig. 1*c* can no longer be recovered and is lost to the phonon system as heat. The rate of energy loss may thus be expressed in the form

$$dW/dt \propto W_p |\gamma_{12} - \gamma_{21}| = W_p |df'/dt| \propto |dP/dt| \quad (5)$$

since the excess occupancies f' are directly related to the excess

transition rates. The absolute value signs imply that the energy loss occurs for either sign of the rate of change of f' . Assuming now an alternating electric field at a frequency ω , $E = E_0 \sin \omega t$, we may define a frequency-dependent complex susceptibility $\chi(\omega) = P/E$, implying a phase lag between P and E due to the presence of loss.

The physical nature of this loss may be envisaged by considering the extra energy required from the alternating field to cause the increased transition rate from the relaxed states on reversal of the sense of polarisation. This energy is derived from the phase lag between P and E .

Frequency dependence of the susceptibility

To obtain the energy loss per cycle, W_c , we must integrate the rate given by equation (5) over the half period during which $dP/dt > 0$. During the other half cycle the loss arises on the opposite sites in the pairs whose occupancy is being increased at the time. The loss per cycle is therefore proportional to the amplitude of the polarisation P , but the frequency ω does not appear in the result of the integration. So

$$\chi''(\omega) \propto |\chi(\omega)| = [\chi'^2 + \chi''^2]^{1/2} \quad (6)$$

regardless of the frequency ω . However, this relation can only be satisfied generally if

$$\chi''(\omega) \propto \chi'(\omega) \quad (7)$$

that is, if the real and imaginary components of the complex susceptibility are the same functions of frequency, implying that

$$\chi''(\omega)/\chi'(\omega) = \text{constant with respect to frequency} \quad (8)$$

I note, however, that $\chi'(\omega)$ and $\chi''(\omega)$ must obey the universally applicable Kramers-Kronig relations which in the extended frequency range $(-\infty, \infty)$ may be written in the form of a Hilbert transform

$$\chi'(\omega) = (1/\pi) \int_{-\infty}^{\infty} \frac{\chi''(x) dx}{(x - \omega)} \quad (9)$$

and a similar expression for the real and imaginary parts reversed and with a negative sign in front of the integral. The integral is to be taken here in the sense of the Cauchy principal value.

It is a property of Hilbert transforms that equation (9) and its counterpart can only be satisfied with the condition (7) by the following functions¹⁸:

$$\chi''(\omega) = A \operatorname{sgn} \omega |\omega|^{n-1} \quad (10)$$

and

$$\chi'(\omega) = A \tan(n\pi/2) |\omega|^{n-1} \quad (11)$$

where A is a constant and the exponent of the power law satisfies the condition $0 < n < 1$. This implies that the ratio (8) satisfies the condition:

$$\chi''(\omega)/\chi'(\omega) = \cot(n\pi/2) = \tan[(n-1)\pi/2] \quad (12)$$

so that the ratio of the real and imaginary parts of the susceptibility is directly related to the exponent n .

The upper frequency limit for the applicability of the relations (10) and (11) may be expected to be $1/\tau_0$, the equilibrium transition frequency, since beyond this limit the polarisation would cease to respond to the applied field, just as in the case of the Debye dipolar or hopping processes beyond the reciprocal relaxation time. At the lower end of the frequency spectrum, the ultimate requirement is that the loss must go to zero as ω tends to zero, so that $\chi''(\omega) \propto \omega$ at very low frequencies. In many non-polymeric systems this region is not accessible experimentally because of the onset of d.c. conduction which dominates the dielectric loss. The expected frequency dependence of loss for a single set of hopping pairs is therefore as shown in Fig. 2.

Temperature dependence of loss

Up to this point the analysis was deliberately confined to the discussion of the frequency dependence of dielectric loss, since this could be obtained with the minimum of specific assumptions relating to the relaxation mechanisms. An extension of the

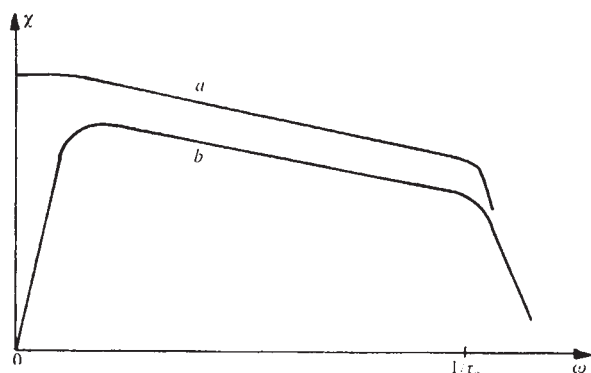


Fig. 2 The frequency dependence of the real and imaginary parts of the complex dielectric susceptibility, $\chi(\omega) = \chi'(\omega) - i\chi''(\omega)$ showing the 'flat' loss accompanied by a similar frequency dependence of χ' and the cutoff at high frequencies above the reciprocal equilibrium transition time, $1/\tau_0$. a, $\chi' \propto \omega^{n-1}$; b, $\chi'' \propto \omega^{n-1}$.

analysis to cover the dependence of loss on temperature would require more detailed assumptions about the nature of the hopping transitions than we are able to make at present.

It may be stated, however, that the temperature would be expected to affect several parameters relevant to the present analysis. The equilibrium transition rate $1/\tau_0$ is certainly thermally activated and the polarisation relaxation time τ_p might be likewise, although the extent of their dependence on temperature may be quite different. Since the relative magnitudes of τ_0 and τ_p determine the applicability of the present analysis, equation (3), as distinct from the conventional Debye-like hopping, some of the contributing mechanisms may be either brought in or eliminated according to the prevailing circumstances.

Distribution of hopping parameters

The idealised model used so far in the present analysis is unrealistic in several important respects. Any real hopping system must contain a distribution of hopping parameters and it may also involve spatial distributions of centres which make possible multiple sequential hopping. In addition, the spatial distribution of the axes of the hopping pairs is normally isotropic so that the effective field is not E but $E \cos \theta$, where θ is the polar angle of the pair with respect to the field E .

The point to be noted in the present analysis is that in the presence of several sets of hopping parameters, or even of a continuous distribution, we are summing flat-loss characteristics instead of summing loss peaks. The final shape of the $\chi''(\omega)$ curve is therefore much less sensitive to the nature of the distribution of hopping parameters than would be a sum of Debye-like loss mechanisms. Of special significance is the point that the range of hopping relaxation times τ_0 does not have to correspond to the experimentally observed range of validity of flat-loss power-law dependence on frequency.

It can easily be envisaged that one of the principal effects of the presence of a distribution of hopping parameters may be an increased temperature dependence, as some of the more strongly activated mechanisms become progressively 'frozen out' with falling temperature.

Discussion

Here I have proposed a hopping model in which the carriers suffer an almost frequency-independent loss of energy arising from a relatively slow relaxation of polarisation in the dielectric matrix in which they hop. This should be contrasted with the frequency-dependent loss normally associated with hopping conduction in nonpolarisable media and also with dipolar

rotations, both of which give rise to characteristic loss peaks as functions of frequency.

One direct consequence of the present model is the fact that both the real and the imaginary parts of the complex dielectric susceptibility $\chi(\omega)$ have the same frequency dependence. The power-law dependence given by equations (10) and (11) then follows as a natural consequence of the Kramers-Kronig relations, leading to a flat frequency response even in the presence of only one single set of hopping parameters. This means that in the present model it is not necessary to invoke any special distribution of hopping or dipolar relaxation times to explain the very widely experimentally observed flat frequency dependence of dielectric loss, as would be the case with the summation of individual Debye-like loss peaks. In particular, it is not necessary to postulate sequential hopping over many sites distributed randomly in space and in energy, as is clearly the case with d.c. conductivity. This makes it much easier to understand the similarity of the experimentally observed behaviour of relatively thick and of very thin samples. It is also clear that the dielectric loss has nothing to do with the d.c. conductivity σ_0 of the material, since they are due to essentially different processes. The lack of dependence of loss on hydrostatic pressure is also understood in terms of the insensitivity of the polarisation energy W_p to pressure, as opposed to the strong dependence of the hopping probabilities themselves, especially the most difficult ones determining the d.c. conductivity.

The present analysis is deliberately confined to a very general model, making the minimum of assumptions about the specific properties of any particular model under consideration. I feel that when we are confronted with the case of a very widely observed behaviour, the first task is to show that this behaviour is consistent with a model postulating only a very generally applicable property—in the present instance the existence of a slow relaxation of polarisation in the dielectric matrix in which charge carriers move by hopping.

A comment is indicated about the relation between the approach proposed in the present paper and the earlier theories of sequential hopping^{3-5,9}. These theories may well be applicable to certain materials which have the required density and distribution of localised levels, for example chalcogenide glasses in which there is also a reasonable correlation between the d.c. and a.c. conductivities. I suggest, however, that the present model offers a wider framework in which it is possible to understand the observed behaviour of many materials to which the other theories could not be applied so easily.

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Chemical and biological evolution of a nucleotide-binding protein

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Three-dimensional alignment of the common nucleotide binding structure in dehydrogenases, kinases and flavodoxins permits the recognition of homologous amino acids when sequence comparisons alone would fail. Minimum base changes per codon can then be used to measure evolutionary distances which suggest that this structure was present during precellular evolution.

A COMMON structural domain¹ whose function is to bind nicotinamide adenine dinucleotide (NAD) has been found in lactate dehydrogenase (LDH)^{2,3}, in soluble malate dehydrogenase (sMDH)⁴, in liver alcohol dehydrogenase (LADH)⁵ and in glyceraldehyde-3-phosphate dehydrogenase (GAPDH)⁶. Rao and Rossmann⁷ showed that the same structure was utilised to bind flavin mononucleotide (FMN) in flavodoxin⁸⁻¹⁰. They also showed that this structure consists of two smaller units the function of each being to bind a mononucleotide. Buehner *et al.*⁶ proposed an evolutionary tree which traces the incorporation and evolution of the mononucleotide-binding structure in various dehydrogenases and in flavodoxin. Further data on the three-dimensional structures and amino acid sequences of some dehydrogenases and flavodoxins are now available which permits the evaluation of the proposed evolutionary tree more precisely. We show here that the position and sequence of the nodes in this tree are consistent with the molecular data, and that a rough time scale for these events can be proposed.

Independent support for the evolutionary tree comes from a study of redox potentials and the evolution of biological electron transport^{11,12}, suggesting a common origin for NAD and flavin-binding proteins. Furthermore, structures similar to the mononucleotide or dinucleotide-binding protein fragment have been found in phosphoglycerate kinase¹³ (Bryant *et al.*¹⁴ suggest an alternative solution) and tentatively in adenyl kinase¹⁵. Both these enzymes need to bind AMP, ADP or ATP. Similarly a flavodoxin-like structure has been recognised tentatively in rhodanese¹⁶ whose biological function is not fully established but is known to bind flavin FAD FMN and NAD.

Structural alignments

The nucleotide-binding protein discussed above consists of a parallel sheet formed by three extended polypeptide strands. The first two are connected by an α -helix, and the last two by

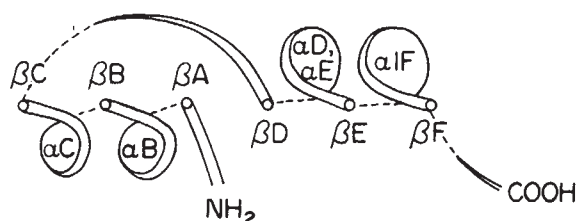


Fig. 1 Diagrammatic representation of a dinucleotide-binding protein. The amino termini of the strands in the β -pleated sheet are closest to the viewer. The dinucleotide binds to the carboxy termini of the strands in the sheet.

either an α -helix or another, less well defined, structure. In dehydrogenases the secondary structural features of the dinucleotide-binding fragment have been termed β A, β B, β C, β D, β E and β F within the β -pleated sheet, while the helices are labelled α B, α C, . . . (Fig. 1). This nomenclature was originally devised for the LDH structure³. Helices α B and α C connect strands β A with β B and β B with β C, respectively. They are on the same side of the sheet. Along the polypeptide chain, the sequence β A, α B, β B, α C, β C forms the first (adenine) nucleotide-binding fragment, while the second (nicotinamide) nucleotide-binding fragment is related by an approximate two-fold axis. The fold of each fragment has the same unique band.

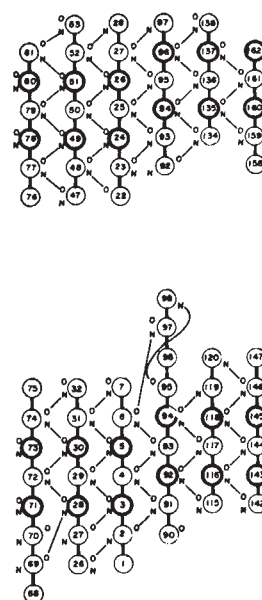


Fig. 2 Hydrogen bonding diagram of β -pleated sheet region in (top) dogfish apo-LDH and (bottom) lobster holo-GAPDH. Amino acids with thick outlines have residues facing into hydrophobic pockets between the β -pleated sheet and α helices. The hydrophobic character of these residues is strongly conserved (Table 1).

There is a greater conservation of the structure of the sheet region than of the helices. For example, in LADH and GAPDH the helix α D is missing, while in LDH and s-MDH it forms part of a flexible loop which undergoes a large conformational change during catalysis. The greater conservation of the parallel pleated sheet region can be seen readily in Fig. 2 where the hydrogen bonding within the sheet has been depicted for dogfish apo-LDH and lobster holo-GAPDH. When these bonds are equivalently aligned, with due regard for the direction of polarity of the peptide chain, then the orientation of the C_β atom in each amino acid side chain must also be aligned. Hence superposition of the hydrogen bonding provides a sensitive method of determining homologous amino acids in equivalent β -sheet regions of different nucleotide-binding fragments. The atomic coordinates of the equivalenced C_α atoms were taken to set up an approximate rotation matrix to superimpose the two molecules being compared. This was then refined to minimise the sum of the square of the distances between equivalenced atoms⁷. With this alignment other residues, not in the sheet, but nevertheless following the same

Table 1 Nucleotide-binding protein fragment comparison of amino acids

Reference to sequence data		β A		α B		β B		β C	
		2	3	3	4	4	5	7	8
		2	3	4	0	7	4	6	8
Dogfish LDH	38	N K I T V V G	C B A V G	M A D A I S	V L M K D L A	D E V A L V D	V M E D K	A K I V S	G K D
		1	8	1	1	2	3	6	7
Pig GAPDH	39	V K V G V D G	F G R I G	R L V T R A	A F N S G K V	D I V A I N D	P F I D L	K A I T I F Q	E E
Lobster GAPDH	40	S K I G I D G	F G R I G	R L V L R A	A L S C G — V	Q V V A V N D	P F I A L	K K I T V F N	E E
Yeast GAPDH	41	V R V A I D G	F G R I G	R L V M R I	A L S R P B A	Z V V A S B B	P F I B L	K K I A T Y Q	E E
		1	2	2	2	2	2	2	2
		9	0	0	1	1	2	3	4
Horse LADH	42, 43	S T C A V F G	L G G V G	L S V I M G	C K A A G — A	A R I I G V D	I N K D K	G A T E C V N	P P
		2	2	2	2	2	2	2	2
		4	5	5	6	6	7	6	3
Bovine GluDH	19	K T F A V Q G	F G N V G	L H S M R Y L	H R F G — A	K C V A V G E	S D G S I		
		5	2	7	3	9	6		
<i>Clostridium</i> MP Flavodoxin	10, 44, 45	1 M K I V Y	6 W S G T G	1 E L I A K G I	2 I E S G	2 K D V N T I N	3 V S D V N		
		1	1	1	1	1	1	1	1
		2	2	3	3	4	5	7	7
		0	7	2	8	7	4	2	9
Subtilisin	46	D V I N M S L	G G P S G	S A A L K A A	V D K A G	V V V V A A A	G N E G S	P S V I A V G	A A
		β D	LOOP	α E	β B	β F			
		8	9	1	1	1	1	1	1
Dogfish LDH	38	A G S	K L V V I T	A G A R Q Q	I F K F I I P N I V K H S P D	C I L E L H P	5 H R I I G B G	6 — C	4
		9	8	2	2	3	8	4	8
Pig GAPDH	39	A G T	A Y V V E S	T G V F	T T M E K A G A H L K — G G A	K R V I I S A	1 L K I V S N A	4 S S C	8
Lobster GAPDH	40	A G A	E Y I V E S	T G V F	T T I E K A S A H F K — G G A	K K V V I S A	1 M T V V S N A	4 S S C	8
Yeast GAPDH	41	G D S	V I A I R S	T G V F	T E L D T A Q K H L K — A G A	K K V V I T A	3 L K I V S N A	3 S S C	3
		2	2	2	2	2	3	1	1
		6	6	6	7	8	1	2	9
Horse LADH	42, 43	G G V	D F S F E V	I G R	D T M V T A L S C C Q — E A Y	G V S V I V G	1 R T W K G A I	1 F G	1
		4	4	5	6	8	1	1	1
<i>Clostridium</i> MP Flavodoxin	10, 44, 45	L N E	D I L I L G	C S A M G D	H E P F I E E I S T K I S G	K K V A L F G	0 C V V V G T P	5 L I	5

The AMP binding fragment above is aligned below with the NMN binding fragment in dehydrogenases and the FMN binding fragment in flavodoxin. LDH Cys 165 and GAPDH Cys 149 form the essential thiol groups of these two enzymes.

Table 2 Comparison of the NAD-binding domain among various dehydrogenases

		1	2	3	4
Dogfish LDH	1		1.12	1.16	1.13
Lobster GAPDH	2	75		1.13	1.00
Horse LADH	3	74	71		1.13
Bovine GluDH	4				

The top right of the matrix shows the minimum base changes per codon for the alignments in Table 1. The bottom left shows the number of those equivalent amino acids whose C α atoms approach each other to within 3.8 Å.

folding sequence could be equivalenced and incorporated into the refinement procedure.

The amino acid alignments shown in Table 1 are thus based on three-dimensional structure, while the character of the amino acids has been determined chemically. Since Rao and Rossman⁷ have pointed out similarity of structure between a part of subtilisin^{17,18} and a mononucleotide-binding fragment, with the aromatic specificity pocket of subtilisin corresponding to the adenine-binding pocket of the dehydrogenases, this comparison has been included in Table 1. Results for individual comparisons among the dehydrogenases are shown as a matrix in Table 2. The resultant alignment of the bound nucleotides is best seen in a series of stereographic diagrams. The C α

backbone of the NAD-binding fragment of LDH has been used as a standard of comparison. In Fig. 3, LDH is compared with the dehydrogenases GAPDH and LADH, in Fig. 4 comparisons are made with phosphoglycerate kinase¹³ and flavodoxin and Fig. 5 shows a comparison of the AMP-binding fragment with the MNN-binding fragment in LDH.

Sequence homologies with GluDH

The alignment of the glutamate dehydrogenase (GluDH) sequences¹⁹ is not based on structure but on amino acid sequence homologies alone. Different methods of testing sequences for homologies in distantly related proteins have been reviewed by Haber and Koshland²⁰, Barker and Dayhoff²¹ and others. The procedure described by Jukes and Cantor²², which depends on counting minimum base changes per codon, has been used here. By far the best alignment was found in comparing residues 1 to 38 in GAPDH with 245 to 283 in bovine GluDH corresponding to β A, α B and β B (Table 1). This corresponds to the most conserved part of the NAD-binding structure and furthermore the character of important amino acids was maintained.

The glycine in position 28 of LDH is strictly conserved as any larger residue would cause steric hindrance to the binding of the ribose ring. The cause of conservation of the glycine in position 33 of LDH is probably related to its position on helix α B immediately opposite the β -pleated sheet. The conservation of LDH aspartate 53 (changed to glutamate in GluDH) at the end of β B must be related to its function of binding the O2' atom in the adenine ribose³. The alternate hydrophobic residues in the β -pleated sheet is also quite outstanding.

Although GluDH is known to have two independent binding sites for NADH or NADPH²³ the sequence comparison



Fig. 3 Superimposed dinucleotide-binding domains using LDH as a standard. The LDH C α backbone is shown in dark as is its NAD coenzyme. Residue numbers are given for LDH only. Corresponding numbers for the other compounds (shown in open bonds) can be derived from Table 1. Comparisons are with (a) GAPDH and (b) LADH.



Fig. 4 Comparison of (a) phosphoglycerate kinase (PGK) and (b) flavodoxin with LDH. Conventions used are described in Fig. 3. The black ball is the Mg^{2+} site in PGK.

could only identify one truly significant portion of the polypeptide chain which compares with the known NAD binding domains in other dehydrogenases.

The alignment shown in Table 1 for GluDH with GAPDH is different from that given by Smith *et al.*²⁴ or the two comparisons given by Engel²⁵, one of which contains Smith's shorter sequence. The alignment given here shows 1.00 minimum base changes per codon with respect to lobster GAPDH as opposed to 1.48 and 1.53 for Engel's relationships. Furthermore, Engel aligns two GluDH sequences with residues 198–261 of GAPDH. However, this region of GAPDH belongs to the domain which supplies catalytic residues and generates substrate specificity, a domain which might be anticipated to be special to GAPDH. Williams and Wilkins²⁶ point out that Engel's alignments were based on doubtful statistical procedures.

Measuring evolutionary divergence

A relative estimate of the time since the divergence from a common ancestor (or node in an evolutionary tree) can be obtained by comparing amino acids of related proteins. Margoliash and Fitch^{27,28} used the amino acid sequence to show homologous alignments between proteins and then the mean change between the sequence can be measured by various scoring procedures to determine evolutionary distance. In this article (except for the GluDH comparisons) three-dimensional structure has been used to obtain alignments, because of its

greater conservation over amino acid sequence. Every pair of structurally equivalent amino acids can then be examined for their genetic relationship.

It is reasonable to assume that the probability of any one amino acid changing to any other is inversely proportional to the minimum number of base changes. Thus there can be 0, 1, 2 or 3 base changes or on the average 1.5 base changes per codon. If the actual genetic code is taken into account, then the average number of minimum base changes required is 1.57 to change any one amino acid to any other. If, further, the natural frequency of amino acids²⁹ is considered, this number changes to 1.51. Whether or not an observed minimum base change per codon is significantly lower than a random variation will depend on its distance from the assumed random level. When additional independent data (such as three-dimensional structures) are available, comparisons are of greater significance than when only sequence information is at hand. Thus a combination of structure and sequence permits the measurement of more distant evolutionary relationships.

The minimum base change per codon can be taken as a measure of relative evolutionary distance, that is the elapsed time since the occurrence of a common ancestor for divergent events. This measure is unlikely to be exactly linear with time if no correction is made for back mutations. Another cause for possible deviation from linearity is that the rate of accepting point mutations may have been different at different times. In general, however, a uniform acceptance rate is a reasonable assumption for a given protein. For GAPDH the observed minimum base change per codon between the pig and yeast

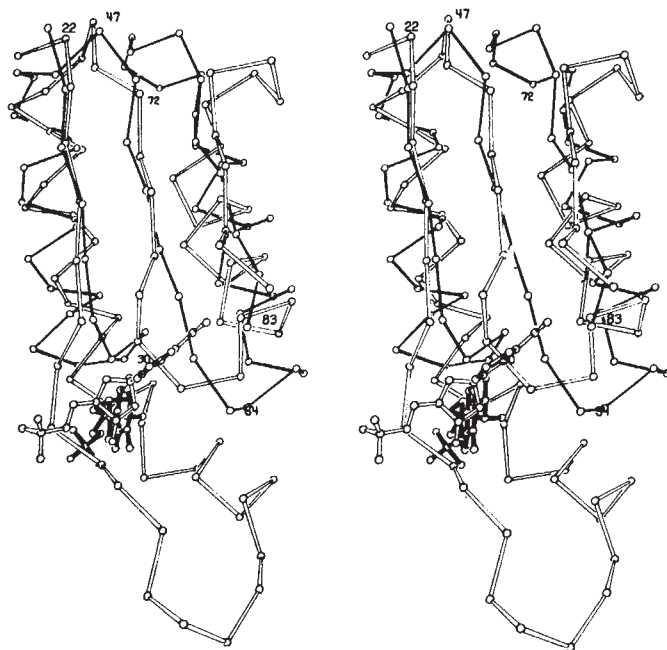


Fig. 5 Superimposed AMP and NMN mononucleotide-binding fragments in LDH. The AMP binding domain is shown with dark bonds and is numbered. The corresponding numbering for the NMN binding fragment can be derived from Table 1. enzyme in the nucleotide binding protein (residues 1-149) is 0.59, but only 0.35 for residues 150-331. Presumably the differing functions of the two domains within a single polypeptide chain¹ impose different rates of evolution. Thus our estimates of divergence among dehydrogenases have been taken only within the nucleotide-binding domains.

A time scale for evolution

The evolutionary tree suggested by Buehner *et al.*⁶ has been

expanded as shown in Fig. 6. Observed minimum base changes per codon are also related to a probable time at each node. The divergence of the mammalian line from Aves can be placed about 3×10^8 yr ago (node 1, Fig. 6), from Arthropoda about 6×10^8 yr ago (node 2, Fig. 6) and from Fungi about 1.2×10^9 yr ago (node 3, Fig. 6)^{30,31}. The presence of the NAD-binding protein in cytoplasmic and mitochondrial dehydrogenases implies that it originated more than 1.5×10^9 yr ago when mitochondria were possibly incorporated into proto-eukaryotes³². Probably, however, this structure is a good deal older as it would have been required in the earliest prokaryotes for glycolysis around 3.2×10^9 yr ago³³. If it is assumed that simple polypeptides and nucleic acids had gained the ability to perform energy transfer steps and primitive copying processes during precellular evolution³⁴ we might expect to find the mononucleotide-binding protein before 3.2×10^9 yr ago. It is also reasonable to assume that it was formed sometime after the age of the Earth 4.5×10^9 yr ago³⁵.

Figure 6 shows that, the larger the age associated with the node, the greater is the minimum base change per codon consistent with a divergent evolutionary process from a single common ancestor. That alternative nucleotide-binding proteins exist is, however, evident in the structures of RNase³⁶ and staphylococcal nuclease³⁷. Nevertheless, it might be anticipated that the basic structure shown in Fig. 1 will frequently be found where there is requirement (especially an old and basic requirement) for binding nucleotides. Examples might be amino acid tRNA synthetases, ribosomal proteins and virus coat proteins. The recognition of this structure by sequence homology or from X-ray structure determinations may also give guidance as to function where none is definitely known.

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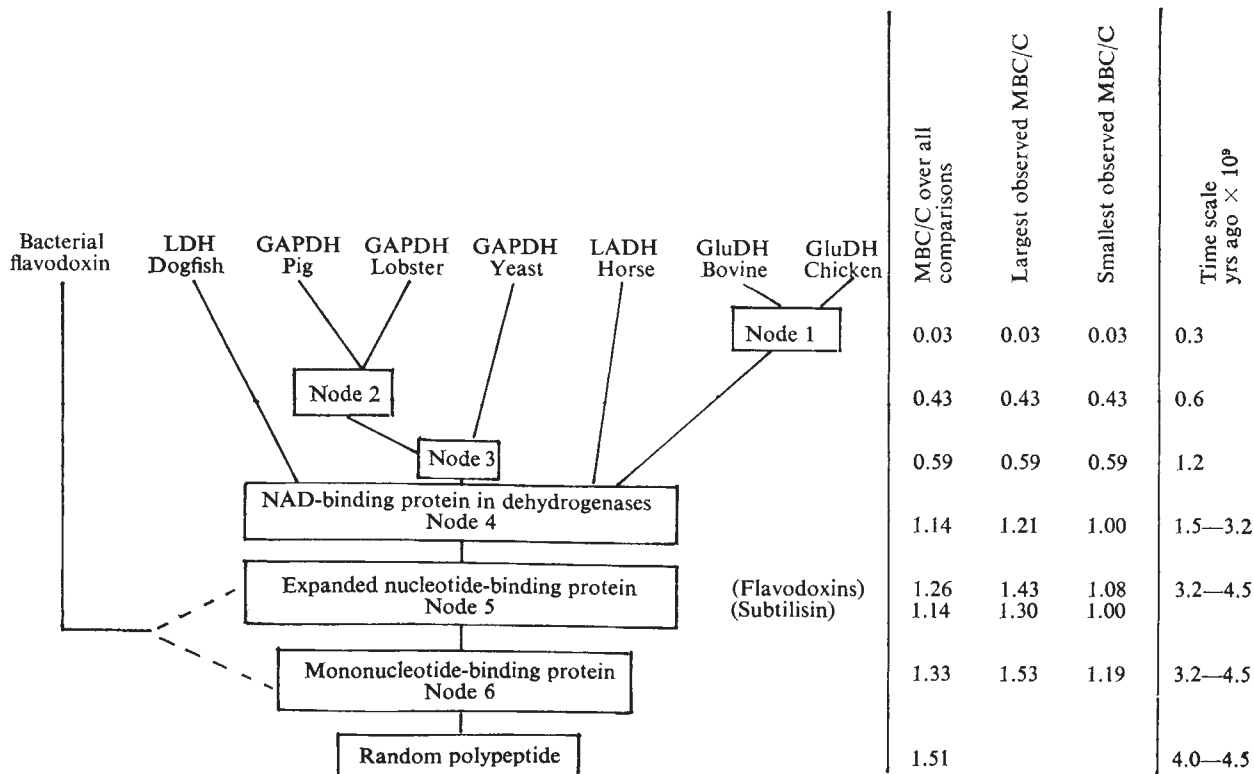


Fig. 6 Evolutionary tree consistent with observed minimum base changes per codon (MBC/C) and possible time scale derived primarily from fossil data.

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Three abundance classes in HeLa cell messenger RNA

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Approximately 35,000 different poly(A)-containing RNA sequences are present in HeLa cell cytoplasm. The sequences are grouped in three distinct abundance classes.

THE amount of DNA per haploid genome in the higher eukaryotes is known to be very large, and this has long posed the question of how much of the DNA encodes mRNA. Up to the present time few serious attempts have been made to answer this question. One problem has been the difficulty experienced in attaining sufficiently high concentrations of RNA to drive hybridisation reactions to completion. The discovery of poly(A) stretches in mRNA¹⁻³ has partially removed this difficulty, since poly(A)-containing RNA can now be separated from rRNA^{4,5} and much higher concentrations of mRNA can be attained. A second difficulty has been the existence of repetitive sequences in the DNA⁶: the transcript of one repetitive sequence can hybridise with related sequences,

producing falsely high results. This problem was solved by Hahn and Laird⁷ and others⁸⁻¹¹ using a technique devised by Kohne¹². Labelled nonrepetitive DNA sequences are isolated using hydroxyapatite, and annealed with an excess of unlabelled RNA. The DNA-RNA hybrids are then recovered on hydroxyapatite. In this way it was shown that about 10% of nonrepetitive mouse DNA was represented in transcripts found in total brain RNA^{7,9,10}. Similarly 0.9% of nonrepetitive *Xenopus* DNA is represented by transcripts in the mature oocyte⁸.

An even more serious problem has been that whatever answer was obtained, it had to be regarded as a minimum. No matter how much RNA was added, or for how long the samples were annealed, the possibility always remained that classes of RNA were present at a lower concentration than the least concentrated sequences observed to react. Such RNA sequences would fail to react significantly with the complementary DNA sequences, and would go undetected. This problem can now be solved in the following way. It is now possible to synthesise a complementary DNA

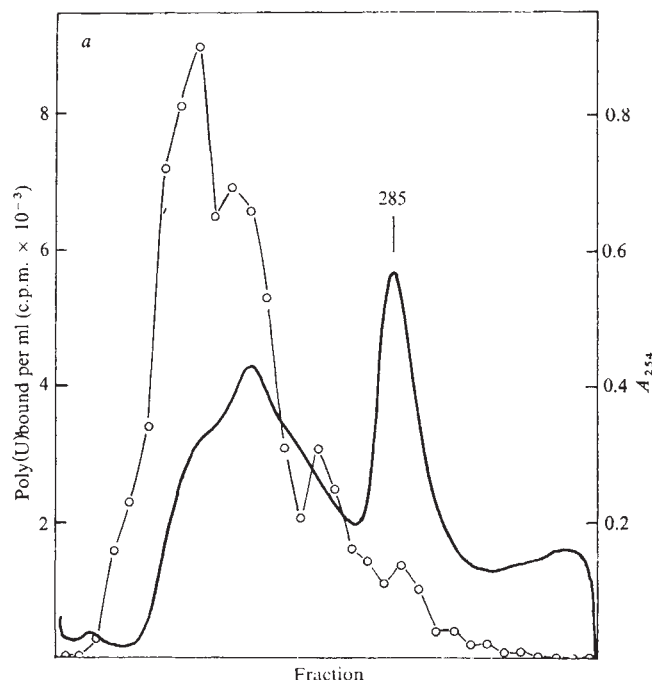
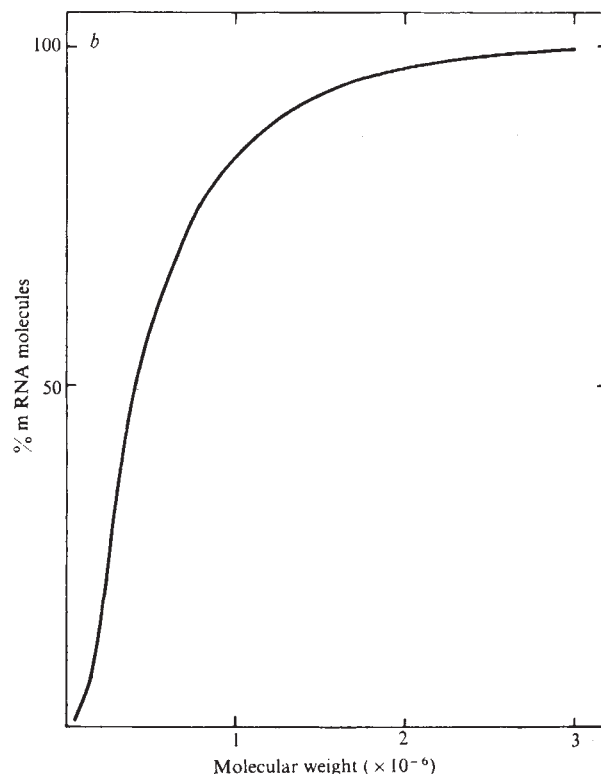


Fig. 1 Molecular weight distribution of HeLa cell mRNA. *a*, Sucrose gradient of enriched mRNA fraction showing A_{254} and results of annealing a portion of each fraction with poly(-U)²¹(o). Sedimentation is from left to right. *b*, Cumulative plot of number of mRNA molecules against molecular weight (calculated according to Spirin²³ and Gierer²⁴). Two gradients like that shown in panel *a* were averaged. Approximately 10^9 HeLa cells were washed and lysed with RSB containing 1% NP-40. Nuclei were separated by centrifugation and the outer membrane fraction was removed²⁵ and added to the cytoplasmic fraction. Cytoplasmic RNA was extracted by a procedure²⁶ modified after Parish and Kirby²⁷ and passed repeatedly over oligo(dT)-cellulose⁴ until free of material which reacted with poly(U)²¹. The poly(A)-containing RNA was eluted⁴ and again treated in the same way. The final eluate from oligo(dT)-cellulose was centrifuged for 16 h at 25,000 r.p.m. and 25°C on a 15–30% sucrose gradient in NTE (0.1 M NaCl, 10 mM tris, pH 7.5, 1 mM EDTA) containing 0.5% SLS (Spinco No. 27 rotor). The mRNA preparations used in this report were prepared by precipitating fractions between 5 and 35S with ethanol, and passing the precipitate, dissolved in 0.3 M NaCl–10 mM Na acetate, pH 5, over a column of Sephadex-SP50 overlying Chelex-100, developed with the same buffer.



transcript (cDNA) on a template of poly(A)-containing mRNA^{13–15}. By annealing this with an excess of the RNA template, we can determine the R_{ot} (product of initial RNA concentration and annealing time) at which the cDNA transcript hybridises completely. The same R_{ot} will be sufficient to obtain hybridisation of all the DNA complementary to mRNA when an excess of unlabelled mRNA is annealed with labelled single-copy DNA. Thus, in principle, the answer obtained should be a definitive one.

The availability of cDNA offers a second important advantage in that the kinetics of hybridisation between cDNA and an excess of unlabelled mRNA are dependent on the sequence complexity of the mRNA^{16,17}. By comparison with a suitable kinetic standard it should be possible to estimate the complexity of the mRNA in favourable cases. When this is so, we have available two independent estimates of mRNA sequence complexity. If they agree, confidence in the estimates will be greatly strengthened.

Preparation of HeLa cell mRNA

The mRNA was prepared by extracting the total cytoplasm of cells lysed with 1% NP-40. Thus, both free and membrane-bound polyribosomes contributed their mRNA populations, together with any mRNA present in the

postribosomal fraction. Poly(A)-containing RNA was prepared using oligo(dT)-cellulose⁴. The evidence at present available^{18,19} indicates that most mRNA contains poly(A). The great advantage of using this method is that it provides a defined, reproducible fraction of the total cellular RNA which is predominantly mRNA. Nevertheless, such preparations contain some 18S and 28S rRNA (Fig. 1). To accommodate this problem, we titrated each mRNA preparation with radioactive poly(U)^{20,21}. Two preparations of HeLa cell mRNA (containing rRNA) bound 8.4% and 8.6% by weight of poly(U). If we take the number-average molecular weight of the mRNA to be 640,000 (2,000 nucleotides) and the average length of the poly(A) to be 150 nucleotides²² the amount of poly(U) bound should be 7.5% by weight (the ribonuclease-resistant complex formed between poly(A) and poly(U) is a 1:1 complex²¹). In practice, we assumed a 10% by weight binding in calculating the mRNA concentration. Minor adjustments are made in the subsequent calculations.

The number-average molecular weight of the mRNA was estimated by annealing sucrose gradient fractions with radioactive poly(U) (Fig. 1). Assuming that poly(A) size is the same irrespective of the size of the mRNA, the number-average molecular weight is readily calculated to be close to 640,000.

Molecular hybridisation

Total HeLa cell mRNA proved to be as efficient as haemoglobin mRNA as a template for cDNA synthesis by avian myeloblastosis virus reverse transcriptase. The reaction was completely dependent upon priming by (pT₁₀) suggesting that synthesis starts at the 3' ends of the mRNA molecules within the poly(A) sequence^{13–15}.

In vast DNA excess hybridisation experiments, labelled HeLa cell mRNA shows a prominent repetitive component (A. Spradling, S. Penman, S. Campo and J. O. Bishop, manuscript in preparation). To estimate the proportion of

repetitive sequences in the cDNA, it was annealed with an excess of unlabelled HeLa cell DNA. Highly labelled non-repetitive HeLa cell DNA was also annealed with unlabelled DNA to provide a comparison (Fig. 2). The non-repetitive cellular DNA behaved as though completely unique, with a $C_{0t\frac{1}{2}}$ of close to $10^3 \text{ mol s l}^{-1}$. The cDNA showed evidence of a 10% repetitive component. The $C_{0t\frac{1}{2}}$ of the second, major cDNA transition is also close to $10^3 \text{ mol s l}^{-1}$.

The hybridisation of the cDNA with an excess of mRNA (template) is shown in Figs 3 and 4. Figure 3 shows a linear-log plot of the data. Three transitions are seen, with midpoints ($R_{0t\frac{1}{2}}$, mol s l^{-1}), of 0.05, 0.9 and 45. That these are real is shown more clearly in Fig. 4. The linear-log plot tends to obscure the inflections between transitions which are more easily seen in straightforward linear plots. Three of these are needed to cover adequately the range of Fig. 3. Figure 4a shows the first inflection clearly, Fig. 4b shows both, and Fig. 4c shows the second clearly. Note the differences in the scale of the three abscissae of Fig. 4.

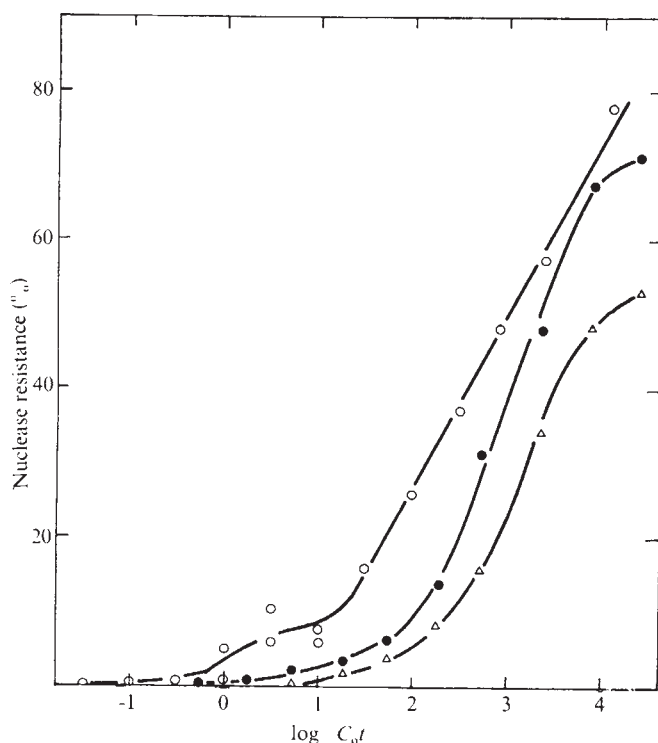


Fig. 2 Renaturation experiments with an excess of unlabelled HeLa cell DNA. The labelled species were (●) nonrepetitive HeLa cell DNA; (○) cDNA prepared against total cytoplasmic poly(A)-containing RNA; and (△) the fraction of nonrepetitive HeLa cell DNA which hybridises with mRNA at a R_{0t} of 350. cDNA was synthesised using as template mRNA prepared as described in Fig. 1 (ref. 28, except that the reverse transcriptase was purified through CM-Sephadex²⁹ and the substrate was ³H-dCTP (13.3 Ci mmol⁻¹)). HeLa cell DNA was purified as described³⁰, sheared at 50,000 p.s.i. in a Sorvall French Press at 10⁻¹⁵° C in 0.3 M NaCl-10mM Na acetate, pH 5, and passed over a Sephadex-SP-50-Chelex-100 column developed with the same buffer. DNA prepared in this way is 300–400 nucleotides long. Labelled DNA ($2.4 \times 10^5 \text{ cpm } \mu\text{g}^{-1}$) was prepared in the same way from cells labelled with ³H-thymidine. Nonrepetitive DNA was purified by annealing to a C_{0t} of 10 mol s l^{-1} and isolating the fraction eluting from hydroxyapatite between 0.12 M and 0.4 M PB (equimolar Na-phosphate buffer) at 65° C. DNA renaturation was carried out as described³¹, but using 0.24 M PEB (equimolar Na-phosphate buffer containing 1 mM EDTA) at 70° C. Samples were diluted to a final DNA concentration of 250–300 $\mu\text{g ml}^{-1}$ and divided into two aliquots, one of which was treated at 50° C for 40 min with nuclease S₁ (ref. 32) in a solution with the final composition: 48 mM PEB, 0.2 mM EDTA, 20 mM NaCl, 30 mM Na acetate, pH 4.5, 0.6 mM ZnSO₄, 0.052 N acetic acid.

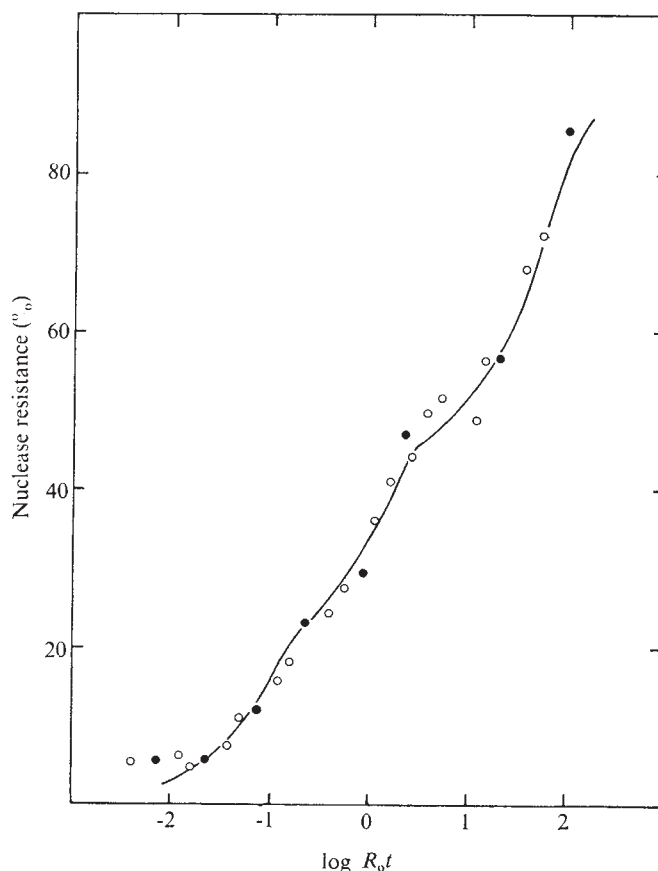


Fig. 3 Hybridisation between cDNA and its mRNA template. Three experiments are shown, two (○) performed with mRNA from cells in spinner culture, one (●) with mRNA from a monolayer culture grown in burlers. In all, five reaction mixtures were used, containing 38, 43 and 295 $\mu\text{g ml}^{-1}$ (○) and 20 and 210 $\mu\text{g ml}^{-1}$ (●) of mRNA, assuming a 10% poly-(A) content. Samples were annealed at 70° C in 0.24M PEB and challenged with nuclease S₁ in the presence of 300 $\mu\text{g ml}^{-1}$ of denatured duck DNA (Fig. 2). The continuous line was drawn using the relationship

$$(d/D_0) = 1 - \sum P_n / e^{k_n t} P_n R_0$$

where D_0 and d represent respectively the initial amount of DNA and the amount duplexed at time t (s) and R_0 the amount of mRNA present (all in mol l^{-1}). The subscript n denotes the transition (1st, 2nd or 3rd). In each transition, P denotes the proportion of the cDNA represented, and k the rate constant of DNA-RNA hybridisation ($\text{l mol}^{-1} \text{s}^{-1}$), given by $k = 0.69/R_{0t\frac{1}{2}}$ (ref. 33). The values taken for the first, second and third transitions were: P , 0.22, 0.28, 0.50; k , 62.7, 2.74, 0.307.

The interpretation of the hybridisation data depends on the availability of a suitable standard. This is provided by the work of N. D. Hastie, M. G. Ferace, K. B. Freeman and J. O. Bishop (unpublished). The $R_{0t\frac{1}{2}}$ of the transition observed when an excess of rabbit haemoglobin α -chain mRNA is annealed with homologous cDNA is $3 \times 10^{-4} \text{ mol s l}^{-1}$. When total ($\alpha + \beta$) haemoglobin mRNA is annealed with homologous cDNA, the $R_{0t\frac{1}{2}}$ is $6 \times 10^{-4} \text{ mol s l}^{-1}$. Since the molecular weight of haemoglobin mRNA is about 200,000 (refs 34, 35) we take $9 \times 10^{-4} \text{ mol s l}^{-1}$ to be the $R_{0t\frac{1}{2}}$ of an RNA with a molecular weight of 6×10^5 .

The data which can be extracted from Fig. 3 are shown in Table 1. The proportion of the cDNA which reacts in each of the three transitions is shown in the first column, and the observed $R_{0t\frac{1}{2}}$ of each in the second. The line shown in Fig. 3 was drawn by summing three ideal first order reactions calculated according to these characteristics. The good fit of the line to the data indicates that it is reasonable to interpret each of the transitions as an

Table 1 Numerical analysis of the data shown in Figs. 3 and 4

Transition	<i>P</i>	$R_{ot\frac{1}{2}}$ (observed)	$R_{ot\frac{1}{2}}$ (if pure)	No. of 640,000 dalton sequences		Molecules of each sequence per cell
				If 90% saturation	If 100% saturation	
1	0.22	0.05	0.015	17	15	8,000
2	0.28	0.90	0.335	370	330	440
3	0.50	45	29.9	33,000	36,250	8

For the definition of *P* see Fig. 3.

ideal pseudo-first-order reaction. (The reaction is pseudo-first- rather than second-order because the RNA is in great excess over the cDNA). If the RNA responsible for any one transition were present on its own, thus contributing all of the RNA used in calculating R_{ot} , the $R_{ot\frac{1}{2}}$ of its transition would be lower. The values of $R_{ot\frac{1}{2}}$ have also been corrected for the 7.5% poly(A) content of mRNA (Table 1, column 3). The numbers of sequences of average molecular weight 640,000 required to generate the three transitions are obtained by dividing the corrected $R_{ot\frac{1}{2}}$ values by 9×10^{-4} (Table 1, column 4). Interpreting Table 1, we would say that HeLa cell poly(A)-containing cytoplasmic RNA contains three distinct fractions: 22% of the RNA comprises only 17 different sequences, 28% comprises 370, and 50% comprises 33,000 different sequences, a total of close to 35,000 sequences. Some qualification is necessary because of the small amount of repetitive sequence found in the cDNA

(Fig. 2) which may contribute disproportionately to the first or second of the transitions seen in Fig. 3. This could affect the absolute value in one case by a factor of 1/3 at the very most.

These numbers were derived by assuming that no more than 90% of the cDNA can form hybrids, as they are measured here. This is true for cDNA prepared against haemoglobin mRNA, where it can be convincingly demonstrated. If the theoretical upper limit were 100%, the numbers would not change greatly (Table 1, column 5). Of course, in the case of any of the transitions, the number-average molecular weight of the RNA molecules need not be exactly 640,000. If so, the calculations would require some adjustment.

The data can also be used to calculate the numbers of copies per cell for RNA sequences of each class. By analogy with complex DNA renaturation patterns⁶ we see that these depend on the observed $R_{ot\frac{1}{2}}$ but not upon either RNA molecular weight or the percentage of the total reaction which each transition occupies. Taking 10^6 HeLa cells to contain 12.5 μ g of RNA of which 5% is poly(A)-containing mRNA, the number of copies per cell equals 400 (observed $R_{ot\frac{1}{2}}$). Thus (Table 1, column 6) there are on average 8,000 RNA copies per cell in the highest frequency class, and about 9 copies per cell in the lowest.

A total of 35,000 RNA sequences of average molecular weight 640,000 represents a total sequence complexity of 2.2×10^{10} daltons. Approximately 20% of the HeLa cell mRNA (prepared in the same way) is repetitive (A. Spradling, S. Penman, M. S. Campo and J. O. Bishop, manuscript in preparation). Accordingly, the overall complexity of the non-repetitive sequences in the mRNA is about 1.8×10^{10} daltons. The analytical complexity of mammalian DNA is 1.8×10^{12} daltons, and of this about 70% or 1.3×10^{13} daltons is non-repetitive. Thus, the cDNA hybridisation kinetic data predict that 1.4% of 'single-copy' HeLa cell DNA is complementary to our mRNA preparation.

Sheared, purified single-copy DNA, highly labelled with 3 H-thymidine, was annealed with enriched mRNA and the double-stranded fraction, containing both DNA-RNA hybrid and DNA duplex, was isolated using hydroxyapatite (HAP). This fraction was dialysed against a low-salt buffer, digested exhaustively with ribonuclease and again fractionated on HAP. This moves the DNA which was hybridised with RNA into the single-strand fraction, while leaving duplexed DNA in the double-strand fraction¹². The results of two such experiments are shown in Table 2. The average of these is 1.05%, in good agreement with prediction. In one experiment the single-copy DNA complementary to mRNA was recovered from the second HAP column and annealed with an excess of unlabelled HeLa cell DNA. Although it failed to renature completely, presumably due to degradation during the very extensive handling it experienced, this fraction clearly did not contain repetitive DNA sequences (Fig. 2).

Number of mRNA species in HeLa cells

One source of uncertainty which remains is the possibility that different mRNA species are not equally transcribed by reverse transcriptase. This is not, however, so serious

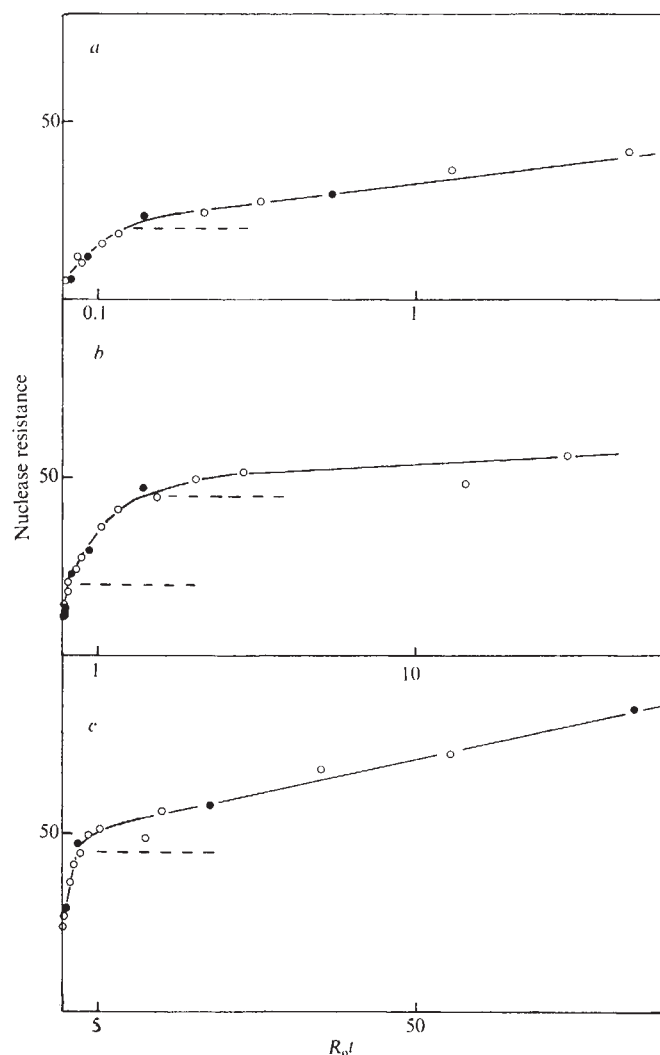


Fig. 4 Linear plots of hybridisation between cDNA and its mRNA template. The data are the same as for the linear-log plot shown in Fig. 3. Dotted lines indicate inflections between transitions.

as might be thought at first sight. Provided that the three frequency classes were transcribed with similar efficiency, even though that efficiency were low, the $R_{ot}\frac{1}{2}$ values would be approximately correct, since they depend on the overall mRNA concentration. If an entire frequency class of mRNA molecules were not transcribed, and if this were the lowest frequency class (in terms of mRNA molecules per cell) the cDNA data would be misleading. This possibility is, however, extremely unlikely. A lower frequency class of RNA molecules which formed a significant proportion of the total would necessarily be complementary to a large part of the single-copy DNA. The good agreement between the kinetic data and the DNA saturation experiments virtually excludes the possibility that such a fraction exists.

Recently, Grady and Campbell³⁷ found that 8.5% of mouse single-copy DNA would form duplexes with total RNA from a mouse cell line, while 15% formed duplexes with RNA from the same line transformed with polyoma. Using a double-reciprocal method to extrapolate the reactions to completion, they estimated that the respective RNA preparations were complementary to about 20% and 30% of the single-copy DNA. If most of the complexity of total cellular RNA is due to HnRNA, the difference between their values and ours is compatible with current ideas on the processing of HnRNA³⁸.

We cannot be sure that all of the poly(A)-containing sequences we have studied are in fact mRNA sequences, although the literature on the subject points strongly in this direction. Assuming that they are, the number 35,000 is much greater than the number of 'genes' estimated by genetic means to be present in the genome of *Drosophila* (for example refs 39–41) and on the same order as the number of protein-encoding sequences estimated by Ohta and Kimura⁴² to be present in the human genome. As far as the human situation is concerned, we may consider three alternative possibilities. (1) HeLa cells may be completely or almost completely derepressed, expressing most of the potential of the human genome. (2) The total potential of the genome may be much greater than suspected, and HeLa cells express only a minor part of it. (3) Human cells have a large number of common functions, and differences between them are determined by relatively few mRNA species: HeLa cells therefore contain the common sequences as a large proportion of their total mRNA population. The answer to these questions will no doubt come from further work of a similar nature with HnRNA and mRNA from cell lines and from tissues.

The different frequency classes of mRNA offer interesting grounds for speculation. In this laboratory, M. S. Campo has obtained evidence which strongly suggests that a small proportion of rat myoblast mRNA contains a high proportion of repetitive sequences. Some of the mRNA

in the higher abundance (molecules per cell) classes may therefore be transcribed from repetitive genes, comparable to histone genes^{43,44}. In HeLa cells, two mRNA populations with different half lives have been described^{45,46}. The more abundant classes of mRNA discovered here may belong to the longer-lived class of mRNA. Lastly, as to function, there are obvious candidates in the protein structure of the cell for more abundant mRNA species, such as actin, tubulin and fibrin, which are present in large amounts in exponentially growing cells, and therefore are very probably synthesised from relatively abundant species of mRNA.

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By applying the same techniques to a *Drosophila melanogaster* cell line (Schneider line 3) we find that it contains a much smaller number of cytoplasmic poly(A)-containing RNA sequences, of the order of 4,000 (M. Izquierdo and J. O. Bishop, unpublished experiments).

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Table 2 Complementarity between nonrepetitive HeLa cell DNA and enriched mRNA

Exp.	R_{ot} (mRNA)	C_{ot} (DNA)	Percentage of total DNA recovered	
			Duplex fraction (First fractionation)	Single-strand fraction (Second fractionation)
1	335	8	4.5	1.2
2	360	8	5.2	0.9

Nonrepetitive HeLa cell DNA was prepared by annealing highly labelled (150,000 c.p.m. μg^{-1}) sheared DNA to a C_{ot} of 10 mol s^{-1} and fractionating by means of hydroxyapatite (HAP)³⁶. Aliquots were annealed with mRNA at 70° C in 0.24 M PEB, diluted to 0.02 M PB and adsorbed to HAP. The duplex fraction was eluted between 0.12 and 0.4 M PB. This was dialysed exhaustively against 10 mM tris, pH 7.5, and then digested for 2 h at 37° C with 20 $\mu\text{g ml}^{-1}$ of RNase. The sample was then adjusted to 0.02 M PB and the fractionation was repeated. The single-stranded fraction was eluted between 0.02 and 0.12 M PB.

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LETTERS TO NATURE

PHYSICAL SCIENCES

Ultraviolet spectra of Capella

In December 1973, during observations from the space observatory Orion 2 on Soyuz 13, three spectrograms of Capella (α Aur, GO III, $m_v=0.2$) were obtained in the wavelength region 2,000-3,000 Å. The spectrograms were made by a wide-angle meniscus telescope of the Cassesgrain system, with an aperture diameter of 240 mm, an equivalent focal length of 1,000 mm, and a 4-grade quartz prism objective. The dispersion of the spectrograph was 170, 280 and 550 Å mm⁻¹, at wavelengths of 2,000, 2,500 and 3,000 Å, respectively.

Spectrograms of the Capella were obtained from exposure times of 15 s (film F-19), 1.5 min (F-20), and 18 min (F-21) (Fig. 1). Even the spectrogram with an

exposure time of 15 s at wavelengths of 2,700-5,000 Å was overexposed. Increasing the exposure time resulted in an extension of the photographic background on the film at the expense of faint stars. This accounts for a regular diminution of the mean photometric amplitude between the background of the film and the darkness, passing from the F-19 record over to the F-21 record.

At spectral resolutions of 8, 14 and 28 Å at wavelengths of 2,000, 2,500, and 3,000 Å, respectively, the Orion 2 spectrograms were originally to be used only for a study of the continuous spectra of stars. Often the quality of the spectrograms, however, proved so satisfactory that stronger

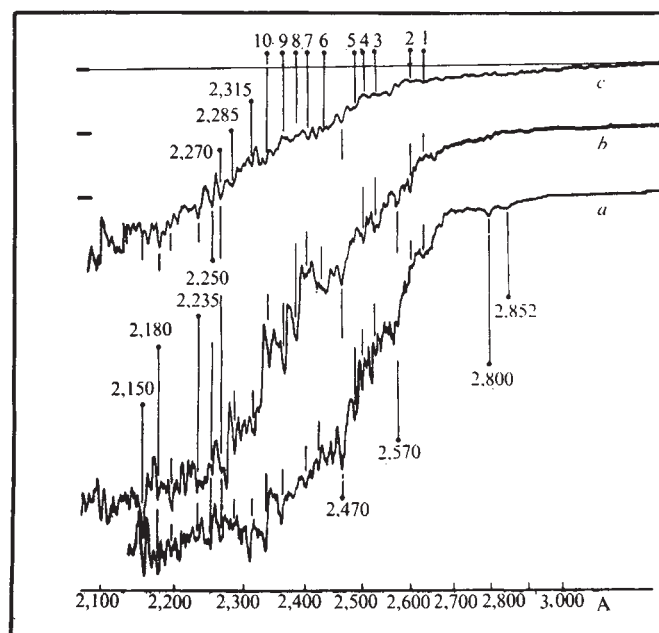


Fig. 1 Microphotometric records of the three ultraviolet spectrograms: a, 15 s (F-19 film); b, 1.5 min (F-20 film); c, 18 min (F-21 film). All wavelengths identified in Table 1. 1-10 as in Table 2.

Table 1 Ultraviolet absorption lines in the spectra of Capella

Observed length (Å)	Proposed identification		
2,852	2,851.6 MgI	2,852.2 MgI	2,851.8 FeI
2,800	2,795.5 MgII	2,802.7 MgII	
2,570	2,570.8 FeI	2,571.0 TiII	
2,470	2,470.7 FeI		
2,365	2,364.8 FeI	MoII	NiII
2,315	2,316.0 NiII	2,316.0 TiII	2,315.6 MoII
2,285	2,286.2 CoII	2,284.1 FeI	2,284.8 CoII
2,270	2,270.2 NiI		
2,250	2,251.2 SnI	FeII	TiII
2,235	2,236.3 CuI	MoII	FeII
2,180	2,180.5 NiII		
2,150	2,148.7 SnI	2,152.2 SnII	FeII

absorption lines or bands could be distinguished. This was the case with the spectrograms of Capella.

The continuous spectrum of Capella extends to about 2,100 Å (Fig. 1). In the interval 2,850-2,150 Å, nearly 10 absorption lines (bands) can be distinguished with certainty (Fig. 1, and Table 1). Strictly speaking, each of these 'lines' is a band resulting from the blending of contiguous absorption lines. There is some doubt about the reality of line 2,150 Å, although it occurs with enough certainty, simultaneously on F-19 and F-20 film.

Moreover, about 10 absorption lines can be marked out less confidently (Fig. 1 and Table 2). In fact, however, the true number of measurable absorption lines exceeds those indicated in both Tables, especially in the region shorter

Table 2 Ultraviolet lines in the spectra of Capella

No. of line in Fig. 1	Observed length (Å)	Proposed identification		
1	2,640	TiII	FeII	
2	2,610	FeII	NiII	
3	2,540	FeI		
4	2,510	FeI	NiI	
5	2,490	FeI		
6	2,440	FeI	MgII	TiII
7	2,405	NiII	MoII	FeII
8	2,385	FeII	MoII	
9	2,365	MoII	MoI	CuII
10	2,345	FeII	NiII	

than 2,300 Å. In some cases even the weak line is distinctly visible in all three spectrograms (although using F-21 film) at wavelengths shorter than 2,250 Å, the short-wave tail is veiled by diffuse light and by the field from Capella itself.

The final location of such lines, however, must await a more thorough examination.

At the wavelengths under consideration (2,800–2,150 Å) the absorption lines in the spectrum of Capella pertain largely to the neutral metals and their ions, which have ionisation potentials of less than 8 eV. These are: FeII, NiII, CuII, MgII, TiII, SnII, SiII, MoII, MnII, and so on.

The 'elevation' in the continuous spectrum at 2,350–2,430 Å, is clearly seen on F-20 film, less clearly seen on F-21 film and only partly seen on F-19 film (Fig. 1). It is unlikely that the elevation results from a blending of the emission lines: rather, it simply forms part of the continuous spectrum. This must lead to the conclusion that there is a large zone of depression on both sides of the elevation, extending to nearly 2,700 Å from the long-wave side, and to 2,100 Å from the shortwave side. Future observations will show whether this conclusion is correct.

A more detailed analysis of the spectrograms will be discussed elsewhere.

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Faraday rotation studies in Africa during the solar eclipse of June 30, 1973

FOUR stations were set up in southern and eastern Africa (Table 1) to observe variations of total electron content during the eclipse of the Sun on June 30, 1973. Each station was equipped with a conventional Faraday rotation recording system consisting of a receiver and a mechanically rotating aerial. We recorded the amplitude fading of the 137 MHz signals from the geostationary satellite, Intelsat IIF3, located at approximately 14°W. The four stations were operating for a total period of 10 d, including June 30, the day of the eclipse.

Chimonas and Hines¹ first suggested that internal atmospheric gravity waves should be generated as a result of the Moon's shadow travelling with supersonic speed during an eclipse of the Sun. In the case of the solar eclipse of March 7, 1970 they suggested that the magnitude of the pressure perturbation would be as much as 10% at an

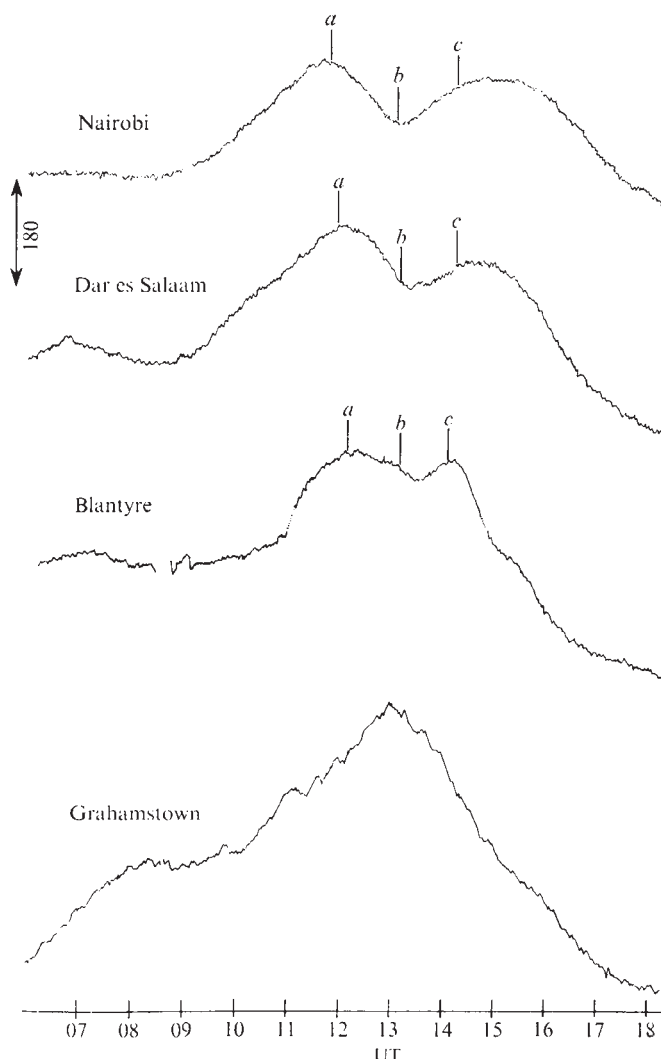


Fig. 1 The variation of the Faraday rotation angle of the 137 MHz transmission received from Intelsat IIF3 at four stations in eastern and southern Africa during the eclipse of June 30, 1973. *a*, Start of the eclipse; *b*, maximum phase of the eclipse; *c*, end of the eclipse.

altitude of 200 km. Davis and da Rosa² observed fluctuations of 1.5% in total electron content on signals from two geostationary satellites, which they tentatively associated with the eclipse. Other workers^{3,4} also observed ionospheric disturbances at other approximately appropriate for eclipse generation. Frost and Clark⁵ conclude that there is insufficient evidence to associate the observations positively with the eclipse. Beer and May⁶ computed the expected paths of bow waves generated by the solar eclipse of June 30, 1973 and concluded that the waves would come to a focus in southern Africa. It had been estimated⁷ that in the region associated with this focusing, the bow wave amplitude should be at least 10 times greater than in California in 1970.

During the 1970 eclipse the directions of the bow waves,

Table 1 Stations established in Africa during the 1973 eclipse

	Station coordinates		Coordinates of the ionospheric point	
	Latitude	Longitude	Latitude	Longitude
Nairobi	1.3° S	36.8° E	1.2° S	32.3° E
Dar es Salaam	6.8° S	39.2° E	6.3° S	34.2° E
Blantyre	15.8° S	35.0° E	14.7° S	30.4° E
Grahamstown	33.3° S	26.7° E	22.2° S	30.8° E

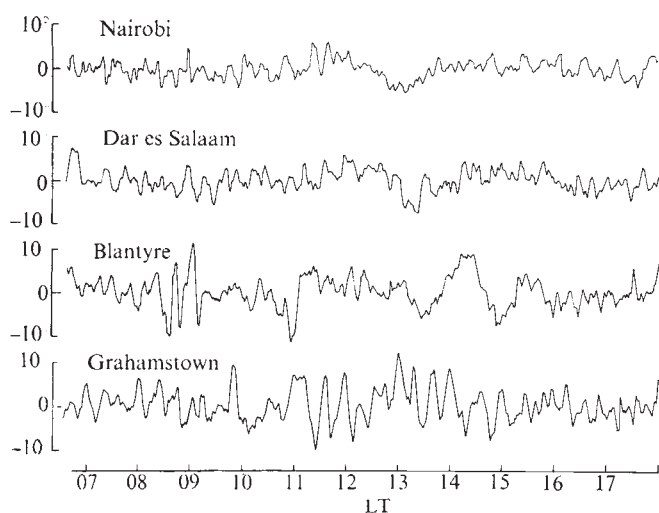


Fig. 2 The raw data after filtering to remove long period changes in the total electron content. The filtered data are shown for a band pass of 5–45 min.

with respect to the local magnetic fields and the radio signal paths, were not so favourable for the observation of travelling ionospheric disturbances induced by the eclipse as in 1973. The greater ionospheric coupling in 1973, and the long duration of the eclipse, encouraged expectations of perturbations of the total electron content of at least 15%.

The positions of the ionospheric points have been calculated for an ionospheric height of 350 km. The Faraday rotation records were read at intervals of 1 min. Totality in East Africa occurred at about 1300 UT, and the estimated time of arrival of the bow wave^{3,6} near Grahamstown was 1500–1700 UT. The expected perturbation of some 15% in the total electron content at Grahamstown could easily have been recorded. The observations, however, show no fluctuations identifiable with the eclipse at any of the four stations (Fig. 1). These results agree with the observations of Schödel *et al.*⁷

The raw data have been filtered to reduce noise and to remove the long period changes (mainly diurnal) in the total electron content (Fig. 2). The results show that travelling ionospheric disturbances with amplitudes of 3% were frequent at Grahamstown; there was, however, no way of associating perturbations on the records with the eclipse at any of the four stations. We conclude that if there were any travelling ionospheric disturbances, induced by the eclipse, they produced variations of less than 1% in the total electron content. Thus, it seems that the disturbances were very much smaller than had been anticipated.

The failure to observe eclipse induced fluctuations of the total electron content under such apparently favourable conditions must raise doubts about the validity of the theories which predict them, and about the interpretation of previous observations. In this connection, we draw attention to the suggestion of Beer⁸ concerning the production of travelling ionospheric disturbances by the terminator travelling at supersonic speed. Titheridge⁹ rarely observed travelling ionospheric disturbances associated with sunrise and sunset.

The times of maximum eclipse and of the beginning and end of the eclipse have been computed by the Nautical Almanac Office for a height of 350 km at the ionospheric points corresponding to the stations at Nairobi, Dar es Salaam and Blantyre. The time delay between the local maximum of the eclipse and the subsequent minimum in the electron content was about 5 min at Nairobi—much shorter than expected. At Dar es Salaam and Blantyre it was around 20 min, which agrees with earlier observations

elsewhere. Klobuchar *et al.*¹⁰ also observed a delay of only 5 min between the minimum in the electron content and the local time of maximum solar observation at Akjoujt (20.1°N, 14.0°W) during the 1973 eclipse. Nairobi and Akjoujt are situated at about 10° on either side of the geometric dip equator in Africa.

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Liquid immiscibility between silicate and carbonate melts in naturally occurring ijolite magma

PORTIONS of the fluids and melts responsible for the crystallisation of rocks and minerals may be trapped and preserved as small inclusions within a crystal, providing much information about the physical and chemical conditions prevailing during crystallisation^{1,2}.

Studies of inclusions in apatites from some East African carbonatites and ijolites³ reveal that carbonate-rich and silicate-rich melts can coexist as immiscible fractions in naturally occurring ijolite magmas. The apatites come from ijolite pegmatites collected from two localities within the Usaki complex of West Kenya⁴. The distinction between primary inclusions (those formed during the actual growth of the crystal) and secondary inclusions (those formed after growth has terminated) was immediately apparent since the primary inclusions characteristically occur as long (up to 150 μm) tubular cavities parallel with the *c* axis of the apatite crystal. Only rarely were small, curved planes of minute (<10 μm) secondary inclusions observed.

Four different primary inclusion types were recognised in the apatites from ijolite pegmatite (U366);

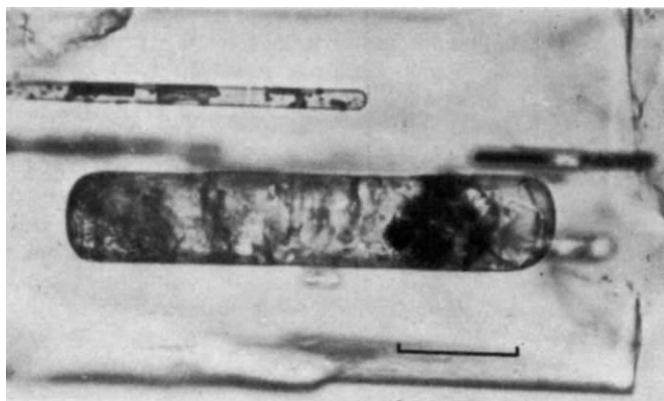


Fig. 1 Large, tubular, multisolid, carbonate-rich inclusion (type A) in apatite from ijolite U366. Most of the colourless, crystalline material within the inclusion exhibit high order interference colours. On the right of the inclusion, several small, black, magnetic, crystalline phases are apparent. Bar = 20 μm .

- (1) Silicate melt inclusions consisting of minute, crystalline specks in a glassy matrix ($n \sim 1.55$) but no vapour bubble.
- (2) Carbonate-rich melt inclusions which consist of at least 60% anisotropic, colourless, crystalline material (Fig. 1) and usually a small amount of aqueous fluid and vapour. This colourless material reacted instantly, dissolving with rapid effervescence, when the inclusions were opened in dilute, acidified (HCl) glycerol on a microscope crushing stage of the type described by Roedder⁵. Most of this material, however, remained insoluble in pure deionised water. This behaviour in acid media and the high order interference colours of these crystals indicate that they are carbonate-bearing minerals. Small crystals of a black, opaque, magnetic ore mineral are also commonly present.
- (3) Nahcolite-bearing inclusions which represent a trapped aqueous fluid phase. These inclusions contain variable amounts of Nahcolite (NaHCO_3 , ref. 6) aqueous fluid, and a CO_2 -rich vapour bubble.
- (4) Gaseous inclusions representative of a vapour phase. These consist almost entirely of highly compressed (sometimes liquefied) carbon dioxide.

In many instances, all four inclusion types occur side by side in the same apatite crystal. Since they are primary³, they represent low temperature equivalents of the trapped portions of phases present in the ijolite magma during the crystallisation of the apatites.

The apatites from ijolite pegmatite (U1256) also contain

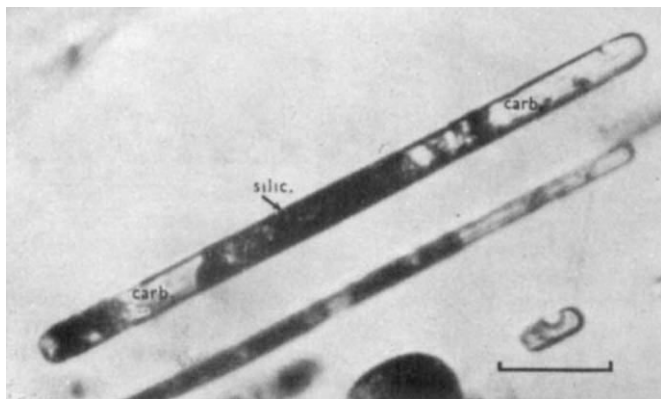


Fig. 2 Complex carbonate-rich/silicate melt inclusion in apatite from ijolite U1256. This inclusion shows a dark, central patch of brown, green and black crystalline phases; predominantly silicate minerals (silic.). The colourless solids are principally carbonate-bearing phases (carb.). Bar = 20 μm .



Fig. 3 The behaviour of the contents of a complex, multi-solid carbonate-rich/silicate melt inclusions on heating (type B from U1256). Bar = 20 μm . *a*, At 23° C, the inclusion as seen at room temperature. Note the presence of a large vapour bubble (V); *b*, At 565° C, melting of the carbonate phase begins; *c*, At 890° C, all the carbonates have melted to a colourless liquid. Small globules of a clear green liquid (one of which is arrowed) have also formed from the dark silicate minerals. Thus, two immiscible liquids are present in the inclusion at this temperature; *d*, At 960° C, complete homogenisation of the carbonate-rich melt and silicate melt has taken place. The vapour bubble, although somewhat smaller in volume, still remains.

silicate inclusions (glassy matrix, $n \sim 1.56$) and carbonate-rich inclusions, but very few of the aqueous, nahcolite-bearing or gaseous inclusion-types. A further type has, however, been recognised in this sample (Fig. 2). These inclusions are intermediate between carbonate melt inclusions and silicate melt inclusions, and consist of various proportions of both silicate and carbonate-rich material. Whereas the individual silicate inclusions and carbonate inclusions represent the separate trapped portions of two distinctly different melts, the complex silico-carbonate inclusions result from the simultaneous entrapping of a portion of carbonate-rich melt and silicate-rich melt. This demonstrates that these two melts coexisted in the ijolite magma as separate phases at the time the apatites crystallised. Heating studies were made on the inclusions using a Leitz 1350 microscope heating stage. The details will be published elsewhere but it is interesting to note the behaviour of the complex silicate/carbonate-rich melt inclusions as they are heated and cooled. Two immiscible liquids were observed at elevated temperatures (Fig. 3c).

When simple carbonate-rich melt inclusions (here called type A) from both samples were heated (U366 and U1256), their crystalline, carbonate-bearing mineral phases began to melt at about 500° C and were almost entirely molten at temperatures between 640° and 750° C. At these temperatures, only a small amount of solid material, including the small, black, opaque, magnetic, crystalline speck, remained together with a small vapour bubble. These phases usually dissolved in the melt at higher temperatures. On cooling, the inclusion contents recrystallised completely and even rapid cooling failed to produce a glass. When the complex silico-carbonate-rich inclusions from U1256 were heated (Fig. 3), however, the colourless, carbonate-rich fraction melted at temperatures between 575° and 640° C but the silicate fraction, seen as small optically isotropic and anisotropic, green, brown and black patches within the inclusion, did not dissolve completely in the resulting melt. Instead, they melted independently to form pale green coloured globules at temperatures between 820° and 900° C. Where these globules occupied up to 20% of the total volume of the inclusions (type B), they gradually dissolved in the carbonate-rich melt at temperatures ranging from about 950° C up to 1,100° C. But in those instances where the globules occupied a volume greater than about 20% (type C), they still remained, coexisting with the carbonate-rich melt, even at 1,100° C (the maximum temperature attainable on the apparatus).

When inclusions of type B were cooled from the temperature at which the two melts had become miscible, the molten contents separated into two distinct immiscible liquid phases; green silicate globules and a carbonate-rich melt (Fig. 4). Continued cooling caused a small portion of the silicate globules to crystallise but most quenched to a pale green-coloured, isotropic glass. The colourless, carbonate-rich melt crystallised at temperatures between about 575° and 500° C.

Cooling of inclusions of type C gave similar results and some of the silicate globules were seen to coalesce into larger globules which subsequently quenched to a glass.

Heating studies on simple silicate melt inclusions were generally unsuccessful owing to leakage. When two small inclusions were heated to 950° C, then cooled, their contents were, however, seen under oil immersion to have quenched to the same pale green-coloured, isotropic glass as the green globules in the complex inclusions.

The exact composition of the two immiscible melts is unknown, but one is certainly a silicate-rich melt and the other carbonate-rich. Other phases such as CO₂ vapour and an aqueous, saline, carbonate-bearing fluid (seen as nahcolite-bearing aqueous inclusions) coexisted with these two melts in the ijolite magma.

Several authors⁷⁻⁹ have suggested that immiscibility may

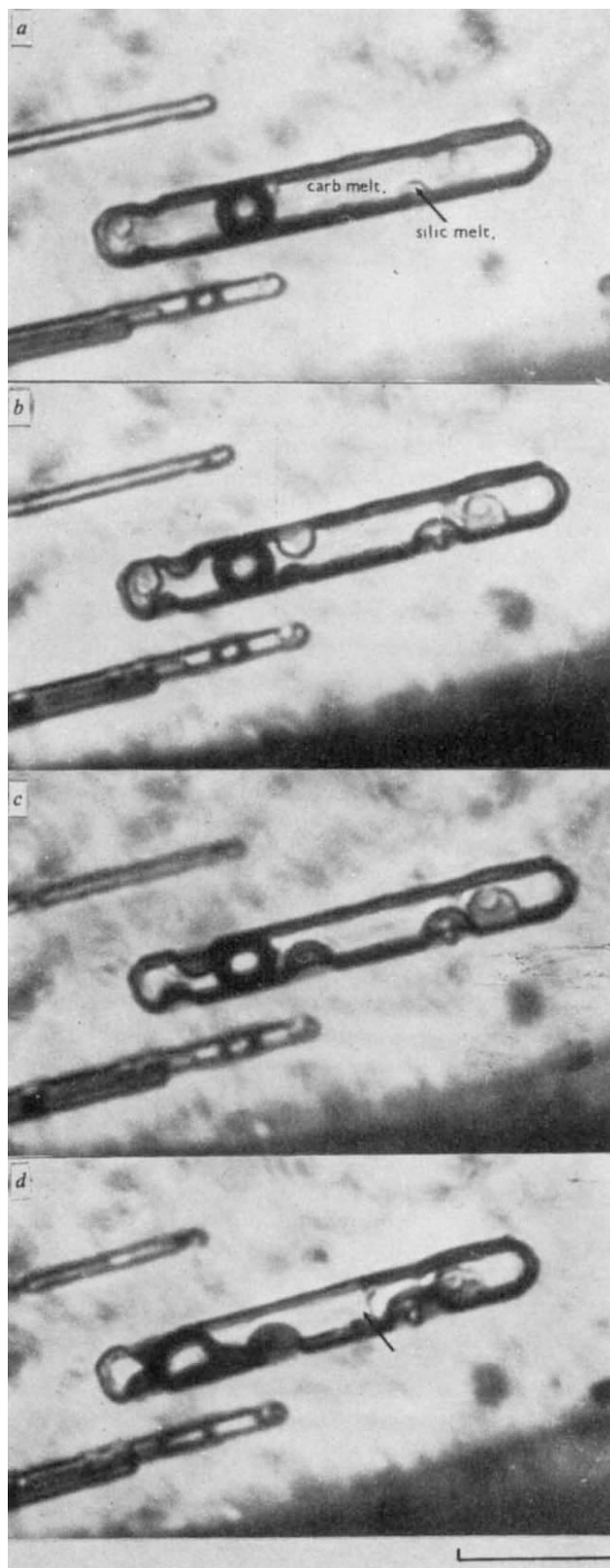


Fig. 4 The behaviour of the contents of the inclusion shown in Fig. 3d, as it is cooled from 960° C. Bar=20µm. a, At 950° C, initial separation (unmixing) of the homogeneous melt takes place. Silicate melt globules (one of which is arrowed) form, leaving a predominantly carbonate-rich melt; b, At 910° C, the silicate globules grow in size and unmixing of the two melts is almost complete; c, At 805° C, some of the silicate globules have coalesced, others begin to crystallise; d, At 575° C, the onset of crystallisation of the carbonate-rich melt (arrowed). The silicate globules have already crystallised or quenched to a glass.

be an important process in the derivation of carbonatitic fluids from parent carbonated silicate magmas. Experimental studies by Koster van Groos and Wyllie¹⁰⁻¹² on selected joins in the system $\text{CaO}-\text{Na}_2\text{O}-\text{Al}_2\text{O}_3-\text{SiO}_2-\text{CO}_2-\text{H}_2\text{O}$ at higher temperatures have confirmed the existence of immiscibility between carbonate-rich and silicate-rich melts in soda-rich synthetic systems, though it does not appear in more lime-rich systems¹³. Since the inclusions studied here represent trapped portions of a natural magmatic system, it is evident that immiscibility between carbonate-rich melts or fluids, and silicate melts can take place in natural ijolitic magmas. These data may be interpreted to show two different possible relationships between carbonatitic and ijolitic melts. First, a parental ijolite magma can, by immiscibility differentiation, produce carbonatite and silicate (?nepheline-syenite) partner magmas; or second, a hyperalkaline silicate parent magma can produce immiscible carbonatite and ijolite partner magmas. The former provides the simplest solution but we believe the field evidence indicates the latter.

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Fig. 1 Transmitted light photograph ($\times 96$) of glass-kaersutite contacts from nodule 1718, Volcan de Tenequia. Kaersutite showing rounded margins is on the left, clinopyroxene is on the right. The glass contains a few microlites and minor olivine. The white areas are glass vesicles.

Nodules containing kaersutite, from the Volcan de Tenequia eruption of 1971, were collected on the southern tip of La Palma, Canary Islands. The wehrlite nodules are seldom greater than 10 cm in the largest dimension, are generally ovoid in shape, and consist of olivine (Fo_{80}), clinopyroxene, opaques and kaersutite. The most notable feature of the nodules is the presence of glass in amounts of 1-5% in several of the nodules. The glass is invariably associated with kaersutite and is highly vesiculated. Petrographic examinations show that the glass is not contamination from associated lavas. The glass-kaersutite relationships are shown in Figs 1 and 2. Melting of kaersutite is indicated clearly by both the rounded edges of the amphibole (Fig. 1) and the cusped shape of kaersutite margins in contact with the glass (Fig. 2). Occasionally, glass is present along olivine-kaersutite margins. Clinopyroxene shows no evidence of involvement in the melting event. Olivine (Fo_{90}) and very small amounts of clinopyroxene have crystallised from the melt, and are clearly distinguishable from the cumulate phases, both in composition and appearance (Fig. 2). The melting seems to be congruent.

Compositions of kaersutite and glass were determined by microprobe analysis of two separate nodules (Table 1). Compared with kaersutite, the glass is enriched in alkalis and alumina, is slightly enriched in silica, and is strongly depleted in magnesia. Iron, titanium, and calcium values are comparable. The compositional differences can only be attributed to the crystallisation of olivine and pyroxene from the melt.

We propose that the melting relationships shown here represent a model for the production of alkali basaltic magma through the melting of kaersutitic amphibole at depth. An important factor in this proposal is the striking similarity in compositions between kaersutites and alkali basalts. This is particularly true when comparing kaersutites to ankaramitic

Kaersutite is a possible source of alkali olivine basalts

STUDIES into the origin of basaltic magmas have led to the belief that the upper mantle contains a hydrous phase, either phlogopite or amphibole, as a source of water and potassium¹⁻³. Here we present data concerning the role of kaersutite, a possible upper mantle phase, in the genesis of alkali basalts. Specifically, we have observed the melting of kaersutite within ultramafic nodules from La Palma. Both the kaersutite and the glass produced through melting show strong compositional similarities to members of the alkali olivine basalt series. The observed relationships strongly indicate that alkali basalts may be derived from a melting event in which kaersutite is the principal participant.

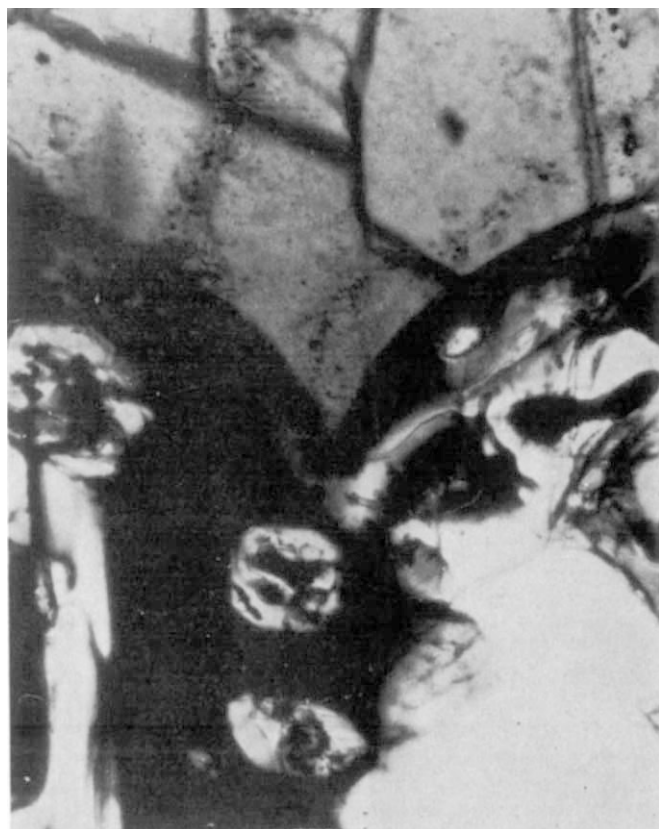


Fig. 2 Transmitted light photograph ($\times 96$) of kaersutite (top), with a cusped shaped margin in contact with glass. A large olivine crystal with a glass inclusion at its centre is at the left. The small equant grains are clinopyroxene. The white area (lower right) is a gas vesicle.

rocks (Table 1) which may represent a parental magma for the alkali basalt series. Ankaramites are characterised by a relatively high K_2O content of $<1-2\%$ with a SiO_2 content of $41-44\%$. Kaersutites, usually noted for a high TiO_2 content, typically have K_2O abundances of $1-2\%$, a value substantially higher than in other aluminous amphiboles.

The observed differences in alumina, magnesia and alkali contents between kaersutite and the associated glass, lie within the variation of rock chemistry shown in the alkali basalt series. Differentiation trends are marked by a rapid decrease in MgO ,

Table 1 Chemical compositions of kaersutite and glass

	*1	2	3	4	5	6	7	8
SiO_2	42.2	44.4	41.4	43.3	43.26	43.2	42.53	43.30
TiO_2	5.25	3.95	3.85	4.14	3.71	3.4	4.78	4.02
Al_2O_3	13.4	15.6	10.3	16.6	13.68	9.69	16.61	16.95
Fe_2O_3	—	—	—	—	3.92	3.66	3.80	3.62
FeO	10.0 ^a	10.8	10.6	10.25	9.39	8.97	7.84	7.70
MnO	0.12	0.18	0.17	0.09	0.22	0.16	0.13	0.06
MgO	11.4	4.05	14.6	4.53	9.22	12.64	5.56	5.72
CaO	12.2	9.61	11.4	10.8	10.28	12.10	10.03	10.49
Na_2O	3.07	4.74	2.93	5.42	3.60	1.59	3.85	4.74
K_2O	1.23	2.77	1.12	2.08	1.47	1.18	1.96	2.34
H_2O	—	—	—	—	—	1.79	1.27	—
Totals	98.87	96.10	96.37	97.21	98.75	99.72 ^b	99.71 ^c	98.94

*1, Kaersutite from xenolith in ash, Volcan de Tenequia, La Palma (No. 1718); 2, glass from xenolith No. 1718; 3, kaersutite, cumulate phase, xenolith from Volcan de Tenequia, La Palma (No. 1719); 4, glass from xenolith (No. 1719); 5, ankaramite, La Palma¹⁰; 6, ankaramite, Vallee de Papenoo, Tahiti-nui¹¹; 7, basanite, Vaitepiha River, Taurapu, Tahiti¹¹; 8, basanite, Las Manchas, La Palma¹²; a, total Fe determined as FeO ; b, analysis total includes $0.67 H_2O$ — and $0.61 P_2O_5$; c, analysis total includes $0.45 H_2O$ — and $0.90 P_2O_5$.

with increases in Al_2O_3 and total alkalis at essentially constant levels of SiO_2 , TiO_2 , FeO and CaO at least to the basanite stage. The glass compositions closely approximate those of basanites (Table 1). As the glass composition is apparently controlled by the crystallisation of olivine, basalt compositions between ankaramites and basanites could easily be produced by fractional crystallisation of this mineral.

There is no evidence that the observed glass-kaersutite relationship actually represents melting of a hydrous phase at depth. Glass has been observed previously in ultramafic inclusions, and has been attributed to incipient fusion of the xenolith immediately before eruption^{4,5}. Similarly, the composition of the cumulate olivine does not indicate a mantle origin. Although the observed melting may, however, have occurred at shallow depths, it is reasonably certain that, compared with likely mantle assemblages, kaersutite would remain the mineral with the lowest melting temperature at depth (ref. 2).

The origin of kaersutites in ultramafic nodules has been generally attributed to the crystallisation of this mineral from alkali basalt magmas under hydrous conditions. It is entirely possible, however, that kaersutite is formed as a product of tholeiitic magmatism as well. Helz⁶ has shown that at $1,000^\circ C$ and $5 K_b PH_2O$, amphibole crystallising from a tholeiitic liquid contains 4.27% of TiO_2 and is comparable in composition to most reported kaersutites. Thus, a widespread kaersutite occurrence may result from the crystallisation and accumulation of the amphibole under hydrous conditions at midoceanic ridges or any site of tholeiitic activity. Gravitational settling of kaersutite may provide a zone enriched in amphibole which could then act as a reservoir for alkali olivine basalt production. At the ridge, melting of some of the stored amphibole, at temperatures in excess of those required for the production of tholeiitic liquids, would account for late-stage alkalic activity. If accumulation has resulted from crystallisation at midoceanic ridges, the lithosphere thus produced would contain at its base a potential source for alkali olivine magma over a large geographic area. Passage of the plate over a 'hot spot' could account for oceanic volcanism at sites far removed from ridges.

Previous arguments for the involvement of amphibole in the generation of basaltic magmas have been based on compositional similarities to basalts⁷⁻⁹, the requirement for a source of potassium¹, titanium⁸, and water, and experimental evidence from whole-rock melting experiments in the presence of vapour^{2,3}. The data presented here strongly suggest that kaersutite is entirely suitable as the principal source of alkali olivine basalts.

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Elastic energy and plate tectonics

A GLOBAL, quadrupolar mechanical analysis of the energy stored in the Earth, based on the concept of a 'reference elastic distortion'^{1,2}, has shown that quadrupolar continental drift, and in particular the westerly polar motion could be understood as resulting from a yielding process which operates as a consequence of the elastic energy in the Earth.

Reduction of elastic energy is, however, not the only driving force available for continental drift; thermal stresses and thermal convection certainly play an important role³. Nevertheless, it is instructive to study the extent to which the elastic energy reduction principle alone can explain the finer details of continental drift. We here give a preliminary report of the calculations which have been made; a more extended account will be published elsewhere.

We have utilised various spherical harmonic analyses of the terrestrial topography⁴⁻⁶ and have taken density fluctuations into account by various degrees of quenching or total vanishing of g fluctuations. The gross features of our results were influenced very little by these effective isostatic compensations. To make things as simple as possible, we have also ignored compressibility and any radial variation of the density ρ or rigidity μ ; these, too, do not seem to alter appreciably our qualitative results, which show a significant correlation between regions of high elastic energy density and regions known to be seismically active. The predicted surface displacements are also in good agreement with accepted directions of continental drift.

The equations for self-gravitating elastic bodies⁷, have a local elastic energy density (EED) term

$$\varepsilon(\mathbf{r}) = \mu u_{ik}^2(\mathbf{r}) \quad (1)$$

which can be replaced by

$$\varepsilon(\mathbf{r}) = \mu [u_{ik}(\mathbf{r}) - u_{ik}^{(0)}(\mathbf{r})]^2 \quad (2)$$

$$u_n(\mathbf{r}) = (R/2g) \{ [(n+2)/(n-1) - ((n+3)/n) (r/R)^2] \times \nabla \Phi_n^{\text{eff}} + (2r/R^2) \Phi_n^{\text{eff}} \} \quad (3)$$

$$k_n = \{ 1 + (\mu \rho g R) / ((2n^2 + 4n + 3)/n) \}^{-1} \quad (4)$$

$$\Phi_n^{\text{eff}} = k_n \Phi_n + (1 - k_n) \Phi_n^{(0)} \quad (5)$$

where u_{ik} is the strain tensor, ($= \partial_k u_i + \partial_i u_k$); $u_{ik}^{(0)}$ is the 'reference' strain tensor which, for simplicity, we assume to be

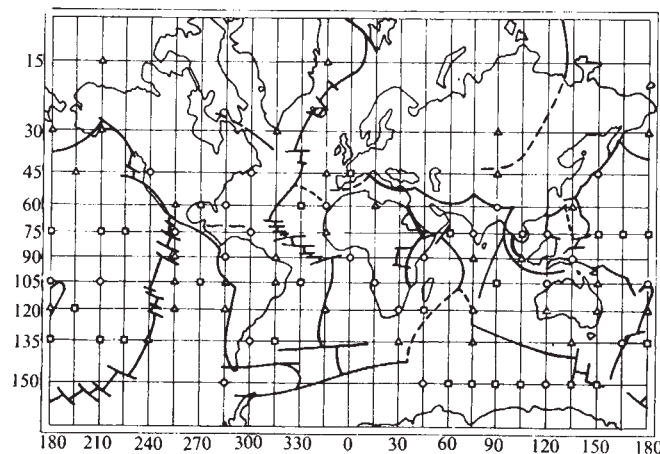


Fig. 1 The movement of the Earth's crust. The arrows represent du_i/dt as calculated from equation (13) in the special case that $\beta = 0$. A solid arrow indicates that du_r/dt is negative, corresponding to crustal sinking; a dotted arrow indicates that du_r/dt is positive, corresponding to crustal rising. Background map taken from Stacey, F. D., *Physics of the Earth*, (Wiley, New York, 1969.)

derivable from a reference displacement $\mathbf{u}^{(0)}$ by $u_{ik}^{(0)} = \partial_k u_i^{(0)} + \partial_i u_k^{(0)}$. Again for simplicity, we assume that $\mathbf{u}^{(0)}$ represents the effect of an effective 'reference potential' $\Phi^{(0)}$ on the fluid body. That is, equation (2) is the elastic energy density for a self-gravitating body which froze when an external potential $\Phi^{(0)}(\mathbf{r})$ was acting on it, and is now distorted differently as a result of the replacement of $\Phi^{(0)}$ by the actual current potential Φ . Expanding all quantities in spherical harmonics, and denoting by subscript n any quantity belonging to the harmonic manifold of order n , the modified equations can be solved to obtain

$$\mathbf{u}_n(\mathbf{r}) = R/2g \{ (n+2/n - 1 - n+3/n) (r/R)^2 \} \nabla \Phi_n^{\text{eff}} + 2r/R^2 \Phi_n^{\text{eff}} \quad (3)$$

where $n \geq 2$. The $n=0,1$ terms only determine the volume and centre of the initial sphere.

Where R and g are the radius and free fall surface acceleration, k_n is related to the n th order Love number⁸

$$k_n = [1 + (\mu/\rho g R) (2n^2 + 4n + 3)/n]^{-1} \quad (4)$$

and

$$\Phi_n^{\text{eff}} = k_n \Phi_n + (1 - k_n) \Phi_n^{(0)} \quad (5)$$

For $\Phi_n^{(0)} = 0$, that is, for departures from spherical shape, equation (5) gives the well known results, whereas with $\Phi_n^{(0)}$ present, the body behaves as if fluid is under a potential which is a weighted average (with weight $k_n < 1$) of the reference and actual potentials.

For actual computation, it is easier to expand, in the usual way,

$$\Phi_n(\mathbf{r}) = gR(r/R)^n \sum_m \varepsilon_n^m y_n^m(\theta, \varphi) \quad (6)$$

$$\Phi_n^{\text{eff}}(\mathbf{r}) = gR(r/R)^n \sum_m \eta_n^m y_n^m(\theta, \varphi) \quad (7)$$

$$\Phi_n^{(0)}(\mathbf{r}) = gR(r/R)^n \sum_m \varepsilon_n^m(0) y_n^m(\theta, \varphi) \quad (8)$$

and the surface radial displacement, which is the terrestrial topography, can be expanded to give

$$u_r(R, \theta, \varphi) = R \sum_{n,m} u_n^m y_n^m(\theta, \varphi) \quad (9)$$

Equation (5) is then transformed to give

$$\eta_n^m = k_n \varepsilon_n^m + (1 - k_n) \varepsilon_n^m(0) \quad (10)$$

and from equation (3):

$$\eta_n^m = -[2(n-1)/2n+1] u_n^m \quad (11)$$

Given η_n^m from the topography analyses and knowing ε_n^m from the actual external potentials, both $\varepsilon_n^m(0)$ and, therefore, the displacement $\mathbf{u} - \mathbf{u}(0)$, which causes the strain which determines the elastic energy density, can be calculated. Note that $\mathbf{v}_n = \mathbf{u}_n - \mathbf{u}_n(0)$ is, again, the displacement of an equivalent fluid body, acted upon by a potential $k_n(\Phi_n - \Phi_n^{(0)})$.

The variables controlling the irreversible release of elastogravitational energy are the $\varepsilon_n^m(0)$ values. In general, the total elastogravitational energy can be written, for $\varepsilon_n^m = 0$, as a sum of squares of the $\varepsilon_n^m(0)$; thus the various processes will act in such a way as to decrease the latter on the average. (Of course, over short time scales some of these could grow at the expense of others, as long as the total energy is decreasing.) The energy reduction process manifests itself in real body and surface motion, as shown by equation (9)–(11). Such a motion, which we shall tentatively consider as a form of continental drift, will depend strongly on the exact way in which the various $\varepsilon_n^m(0)$ values change. In order to try to understand this, we have assumed that the important physical processes are local, so that:

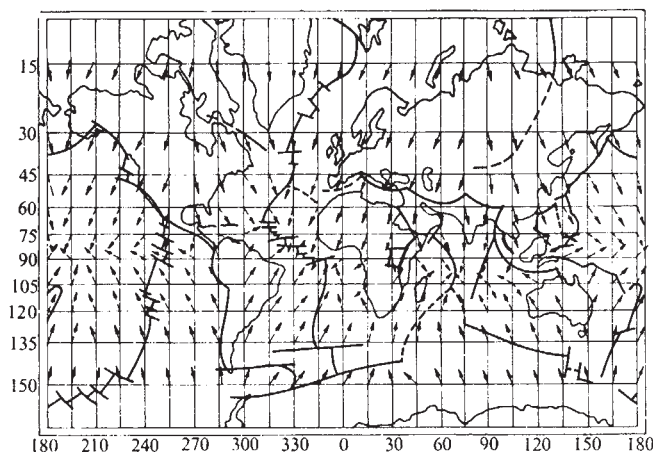


Fig. 2 A plot of EED maxima as calculated with the spherical harmonics coefficients of the Earth's topography in the presence of the Earth's rotational potential⁶. The figure is drawn for a spherical shell at 0.95 Earth radius. Triangles, latitudinal maxima (along a given latitude); boxes, longitudinal maxima (along a given longitude); circles, maxima in both directions. The background heavy lines represent known regions of seismic activity. Background map taken from Stacey, F. D., *Physics of the Earth* (Wiley, New York, 1969.)

$$d/dr[u_n(r) - u_n^{(0)}(r)] = -\alpha' [u_n(r) - u_n^{(0)}(r)] \exp[\beta \epsilon(r)]$$

with suitable values of α' and β . This expression is equivalent to (with $\Phi_n = 0$):

$$d/dr u_n(r) = -\alpha u_n(r) \exp[\beta \epsilon(r)] \quad (13)$$

The actual process of strain release described is considerably more complicated.)

We can apply our formulation to a simple model study—the behaviour of a self-gravitating sphere deformed by

$$\begin{aligned} u_r &= 0 & \text{for } \theta \geq \theta_0 \\ u_r &= -aR(1 - \cos(\theta - \theta_0)) & \text{for } \theta < \theta_0, \end{aligned} \quad (14)$$

where θ is the spherical latitude. This is a 'one-ocean' model of the Earth; θ_0 is the half angular width of the ocean; R the terrestrial radius; and a is a parameter specifying the depth of the ocean, chosen so that the centre of gravity of the deformed body is not too different from the undeformed body.

For this model, we have chosen θ_0 to be 60° , and examined both the EED distribution pattern and the displacement from the reference sphere (a sphere having the same mass and the same centre of gravity as that under consideration). We find that:

(1) the elastic energy is a maximum (of height 10 in arbitrary units) at the centre of the ocean, and falls smoothly to a minimum of 0.65 at 46° , rising again to a maximum of 3.2 at 64° , just off the coast; it falls smoothly again to a low of 0.06 at 118° and finally rises to a maximum of 1.16 at 180° .

(2) the radial component of the displacement vector from the reference sphere $u_r = \sum_{n \geq 2} u_{nr}$ is negative from 0° to 48° ,

positive from 50° to 112° , then negative again from 114° to 180° .

(3) the θ component of the displacement vector from the reference sphere, $u_\theta = \sum_{n \geq 2} u_{n\theta}$, is zero at both poles,

positive from 0 to 78° and negative from 80° to 180° .

We therefore infer that:

(1) it is reasonable to associate regions of maximal elastic energy density with seismically active regions. If our model ocean is regarded as a first approximation for the Pacific Ocean, then we predict seismically active regions in the centre of the ocean, (the Hawaiian Islands), the coasts, (Japan, South-east Asian coasts, the Indonesian islands, and the American Pacific coast) and opposite the ocean centre (the Middle East). These regions are, indeed, seismically active.

(2) Fault lines and plate boundaries can be induced inside the continental land mass as a result of the strain created by the ocean. If we assume, that $\bar{u} \propto \Delta u$ on the basis of equation (13), we would expect fault lines at 50° and 114° , and a plate boundary at 80° . Moreover, the ocean tends to close in order to reduce the overall strain energy.

A second model study was devoted to the investigation of possible interference effects between two oceans, ocean A and ocean P, which are both described by expressions of the form of equation (14). Their maximum depths were chosen to have the ratio 4:7. Ocean A has a half-angular width of 25° and P has a half-width of 75° . The angle between the centres of A and P was chosen as 120° . We found that maxima in EED still exist on the coast and centre of P, and on the far side of the coast of A away from P. There is no EED maximum diametrically opposite to the centre of P because it is too close to the far side of the coast of A. This model shows that interference is quite important. Of course, this model is too rough to match the actual description of the Atlantic and Pacific Oceans.

In the first spherical harmonic analysis of the Earth's lithosphere and hydrosphere⁴, and to a certain extent, in subsequent analysis^{5,6}, the Mid-Atlantic Ridge was not prominently reproduced. As a result, we expect that our calculation of EED and displacements in the Atlantic region is less meaningful than the corresponding calculation for the Pacific Ocean. Using the various sets of spherical harmonic coefficients of the Earth's topography, we have calculated the EED distribution in various spherical shells in the Earth at distances of 1.0, 0.975, 0.95, 0.925, 0.9, 0.85, 0.8, 0.7, 0.6, 0.5, and 0.4 Earth radii for regions of 15° by 15° . We have carried out calculations both with and without the Earth's rotational potential. In each case, we have scanned for latitudinal maxima of the EED (along a given latitude) and longitudinal maxima of the EED (along a given longitude) for each spherical shell. We have associated regions of maximal EED with seismically active regions. Also, at a given angular coordinate, we searched for maxima in the function $r^2 \epsilon(r)$ along the radius. We have associated such maxima with the 'focal points' of seismic regions. We have also calculated the displacement vectors

$$\mathbf{u} = \sum_{n \geq 2} \mathbf{u}_n \text{ and } \mathbf{u} - \mathbf{u}^{(0)} = \sum_{n \geq 2} (\mathbf{u}_n - \mathbf{u}_n^{(0)})$$

According to the simple model expressed by equation (13), the Earth's crust, at a point r , will move with a velocity proportional to the magnitude of $\mathbf{u}$, but oppositely directed to it. Figure 1 shows the plot of du/dr , calculated with the Prey coefficients⁴ with rotation included but without ocean waters as an external load and without isostasy, in the special case that β is 0. It clearly illustrates that oceans tend to close and that mountains tend to disperse.

Space does not permit presentation of all our EED results, which include calculations in each shell for each case considered. All shells in the region between 0.8 to 1.0 Earth radii have an EED pattern fairly close to the experimentally known distribution of seismically active regions, but not quite the same. In most cases, the shells at 0.95 and 0.925 Earth radii give best fits to observed seismic regions. Furthermore, inclusion of the Earth's rotational potential leads to closer agreement with observation. Figure 2 shows the EED maxima distribution in the 0.95 Earth radius shell, calculated with the most recent data⁶ in the presence of the rotational potential.

If only the latitudinal maxima are considered, there seems to be a better fit to the observed seismically active region. A way of understanding this is to suggest that the EED maxima, both longitudinal and latitudinal, are there. Latitudinal maxima are, however, sensitive to Coriolis forces, and so seismic activities may be more associated with latitudinal maxima than longitudinal maxima.

Along a seismic plate boundary, points on the same plate have EED maxima in the same spherical shell or in adjacent shells. In general, EED maxima for points on coastal plates lie closer to the Earth's surface than those on mid-oceanic plates.

A deformed body possesses elastic energy which is gradually released in irreversible processes. It may be possible to understand general features of seismic activity and continental drift as global phenomena related to irreversible strain releases. Although we have not reproduced EED maxima along the Mid-Atlantic Ridge, a ridge does tend to form in the middle of the Atlantic Ocean (Fig. 1). Our EED maxima distribution, in and around the Atlantic Ocean, could well correspond to a situation before the Mid-Atlantic Ridge was formed.

Refinements in the spherical harmonic analysis of the Earth's topography, and inclusion of the effects of isostasy and local variations in μ and g are desirable. Coupling to thermal convection is probably important, as thermal energy seems to be sufficient to move the continental plates³. Core-mantle coupling which would include the effects of the existence of the liquid core may also play an important role. The relaxation process described by equation (13) is obviously too simple and needs to be improved; this will require a better understanding of the solid-state physics of the Earth's crust. We hope to return to these and related questions elsewhere.

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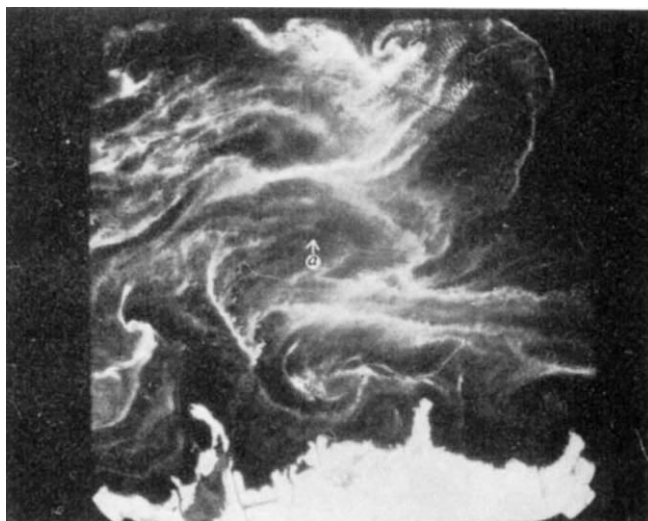


Fig. 1 Infrared aerial photograph of surface algal concentrations in the Upper Arm of Clear Lake, California, taken on May 11, 1973 with an International Imaging Systems Mark I multispectral camera with four 100 mm F. L. lenses and Kodak Type 2424 film. The image is 5 km across. *a*, Wake of boat. Images were simultaneously recorded in red (590-690 nm), green (470-590 nm) and blue (400-470 nm) light. The infrared light used to record this image (730-950 nm) should not be confused with emitted thermal radiation (5,000-14,000 nm). Solar elevation above the horizon was 35°. On the water, dip samples from the first 10 cm of the water column were measured for turbidity and analysed spectrophotometrically for soluble phycocyanin-c and particulate chlorophyll *a* after methane extraction⁶. Secchi disc depths and field notes were recorded at each station. One of us (A.J.H.) devised an aerial photographic technique for surface blooms, which is more available to the average limnologist. A conventional hand-held SLR camera with a through-the-lens light meter, 35-mm lens, standard colour film and a rotatable polarising filter gave a fair impression of the bloom microstructure from a small highwing airplane at an altitude of 2,000 m. Polarised light brought out algal surface patterns but in less detail than the multispectral pictures.

fact that the boat tracks are visible in Fig. 1. Measurements from the boat showed that the lake is virtually free of suspended sediment and that it supports a near monoculture of *Aphanizomenon flos-aquae*¹. Lake surface turbidity, including algae, was only 3 Jackson Turbidity Units. Concentrations of chlorophyll *a* were measured as 14 and 255 $\mu\text{g l}^{-1}$ in two of the photographed areas and we are therefore sure that the pattern resulted from a single species of blue-green algae. Complex patterns such as these cannot be determined using conventional techniques because major changes in algal distribution can occur in a few minutes and the details are invisible from a moving boat. Sky reflections, internal reflections of upwelling light and the disturbance from the boat itself all add to the problem.

Buoyant, gas-vacuolate blue-green algal blooms are a considerable nuisance in Clear Lake. For example, when the Upper Arm of Clear Lake was photographed (Fig. 1), the surface patterns were very different from those in the Oaks Arm. Algae formed dense streaks several metres wide and 10 cm deep and had chlorophyll *a* concentrations of approximately $10^5 \mu\text{g l}^{-1}$ relative to adjacent values of approximately $10^2 \mu\text{g l}^{-1}$. Physical concentrations such as these, if prolonged, would be catastrophic for both algae, which would die, and humans, who would suffer from the smell. Blue phycocyanin-c, a paint-like pigment virtually unique to blue-green algae, is lost from their cells upon death. Even by 09.00 LT the streaks contained up to 500 times more free phycocyanin-c than was observed in nearby areas, indicating considerable decay of the surface phytoplankton. In spring *Aphanizomenon* can fix atmospheric

Remote sensing and lake eutrophication

AN infrared photograph of part of Clear Lake, California (Fig. 1) shows beautiful, complex patterns of blue-green algal blooms which were not observed by conventional limnological techniques. Repeated observations of patterns such as these can be used to chart the surface movement of these buoyant algae and can also be used to help control algal scums in eutrophic lakes.

A considerable amount of microstructure is visible in the photograph, including plume and wave-like concentration. We use the term 'microstructure' because the distances between what are obviously very different concentrations of phytoplankton are only of the order of a few metres, which are small distances relative to the area of the lake (17,000 ha). Although sampling teams on the lake could see some evidence of surface algal concentrations, they did not observe the microstructure at all despite the

nitrogen gas into cellular material and is responsible for up to 50% of the lake's annual intake of nitrogen¹⁻⁴ and therefore, by definition, for much of its eutrophic state. Observations of the destruction of *Aphanizomenon* are important in the control of the extreme eutrophic state of the lake.

Although we believe that most of the observed patterns resulted from *Aphanizomenon* (we also observed a few which resulted from suspended sediment), spectral signatures of the algal patterns varied. On May 11, 1973 algal concentrations were bright in infrared light (Fig. 1); a photograph of the same area in red light provided a similar, but less contrasting, response and photographs in blue and green light showed no patterns. A different spectral signature occurred on June 21, 1973: algal concentrations which were still bright in infrared light, appeared as similar, though darker patterns in blue, green and red light. The spectral signature observed on May 11, however, only occurred in limited areas. Importantly, lysed (dead) algal streaks were bright in all spectral regions. Temporal and spatial signature variations such as these result from any combination of several factors: algal distribution and concentration in the water column⁵, gas vacuolation in the algae⁶, or background turbidities or anomalous concentration effects⁷.

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The main emphasis was on the study of the feasibility of using the favourable properties of synchrotron radiation to achieve high resolution XPS. A resolution of 0.6-1.0 eV (or 0.9-1.5 eV) is achieved in commercially available XPS machines with (or without) monochromatised radiation. We have obtained a total experimental width of 0.42 eV, implying a resolution of about 0.25 eV. Studies in the X-ray region have been limited to the few photon energies determined by characteristic X-ray emission lines (mainly Al $K\alpha_{1,2}$ and Mg $K\alpha_{1,2}$ radiation at 1486.7 eV and 1253.6 eV, respectively, the intensities of which are high enough and the characteristic linewidths of which are small enough to be useful as a monoenergetic X-ray source³. Synchrotron radiation⁴ provides a continuous wavelength spectrum, which for the SPEAR storage ring with a critical wavelength⁴ of $\lambda_c = 4.6 \text{ \AA}$ (at a beam energy of 2.5 GeV) extends down to a fraction of an angstrom ($\approx 1/10 \times \lambda_c$) with considerable intensity. The advantages of using synchrotron radiation for XPS experiments have been reviewed⁵, and parameters of the SPEAR storage ring have been summarised.

In a photoemission experiment the sample is irradiated with monochromatic radiation and a kinetic energy analysis is performed on the photoemitted electrons. Our experimental apparatus, consisting of a beam extractor, a crystal monochromator and an electron spectrometer (Fig. 1). The beam extractor is a 10 m long ultrahigh vacuum pipe ($\Phi = 8$ inches) which is used to channel the synchrotron light from the storage ring out to the experimental area. The beam extractor contains, among other things, instrumentation for collimating the synchrotron beam in both the horizontal and vertical planes. The X rays are allowed to pass two Be foil filters to reduce long wavelength radiation, and through a Be window (250 μm thick), which provides a vacuum tight connection between the ring (10^{-9} torr) and the monochromator (atmospheric He). The crystal monochromator uses two flat Si (220) crystals in the parallel mode and a Si (440) channel-cut crystal in the anti-parallel mode. A third crystal is optional and was not used for these experiments. The first crystal deflects the X-ray beam downwards in the vertical plane and the beam emerges from the second crystal horizontally about 20 cm below the centre-line of the original beam. The monochromator is positioned inside the radiation shielding of the storage ring and must be remotely controlled. The monochromatic X-ray beam (8,000 eV) passes through a hole in the radiation shielding and into the electron spectrometer through a thin (50 μm) Be window, about 15 m from the source. The electron spectrometer consists of an ultra high vacuum (UHV) chamber (base pressure 4×10^{-11} torr) housing an energy analyser, a sample flange, and accessories for the preparation of UHV samples. The energy analyser is a double-pass cylindrical mirror analyser with a channeltron as the electron detector. Standard techniques for fast pulse counting were used for the data acquisition.

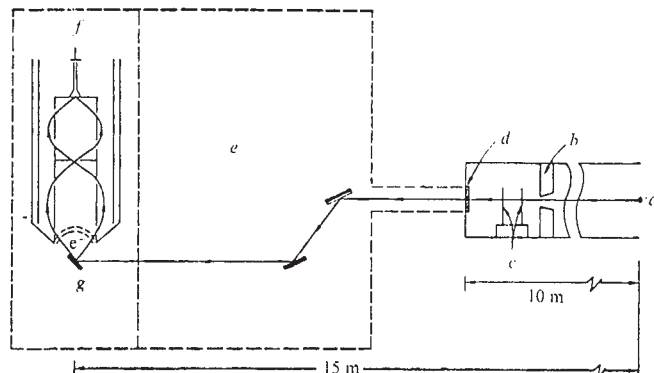


Fig. 1 Schematic figure of the experimental apparatus. a, origin of synchrotron radiation; b, water cooled collimators; c, absorbing Be foils (65 μm); d, Be window (250 μm); e, crystal monochromator (silicon crystals deflect the beam); f, energy analyser; g, sample.

X-ray photoemission spectroscopy

We wish to report the first X-ray photoemission spectroscopy (XPS) experiments performed at Stanford Synchrotron Radiation Project, using synchrotron radiation from the Stanford Positron Electron Accelerator Ring (SPEAR) facility. The photoemission technique has been used extensively in the study of the electron properties of materials^{1,2}.

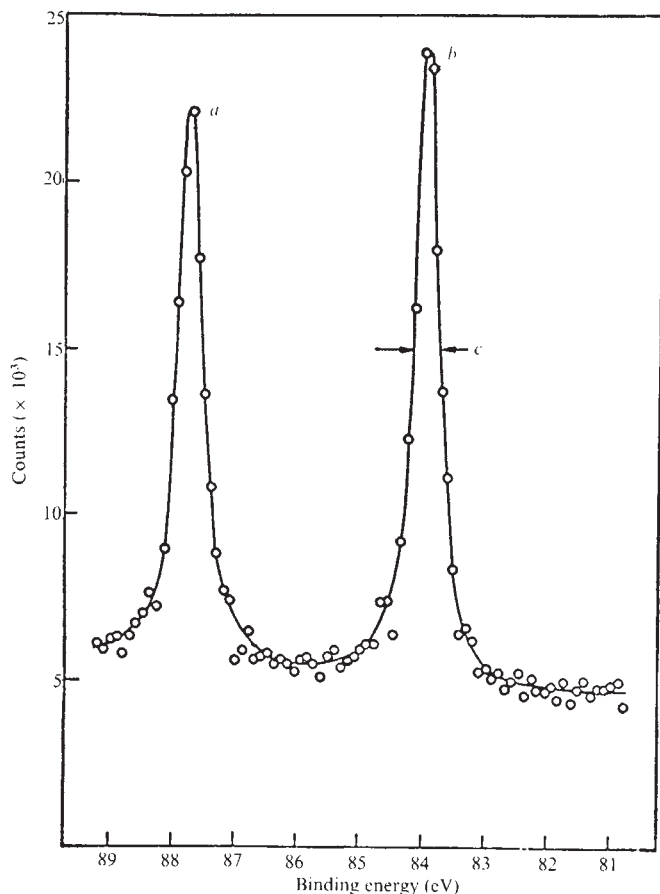


Fig. 2 Electron distribution curve for the 4f doublet of gold. The Fermi level is chosen as the reference level for the binding energy. a, $4f_{5/2}$ peak; b, $4f_{7/2}$ peak; c, 0.42 eV (FWHM).

The performance of our XPS machine was tested by measurements of photoemitted electrons from inner core levels in the noble metals Cu, Ag and Au. The measurements were taken on thin films evaporated *in situ* in the UHV chamber. The observed distribution curve for the well known and extensively examined 4f doublet in Au is shown in Fig. 2. The total accumulated number of counts is plotted against the binding energy of the electrons, using the Fermi energy as the reference level. The counting rate is normalised to an *e*-beam current of 20 mA in SPEAR. During these measurements the ring was run at a beam voltage of 2.5 GeV and an *e*-beam current of 20–40 mA (the beam current typically decays from 30 to 20 mA in 3–4 h, after which the beam is dumped and the ring refilled). The horizontal scale has been calibrated so as to give the binding energy for the $4f_{7/2}$ level, which was reported by Ley *et al.*⁷. The most remarkable thing about the spectrum (Fig. 2) is the extremely narrow width of the lines, demonstrating the high resolution capability of the XPS machine. A linewidth of 0.42 eV at full width at half maximum (FWHM) is obtained from Fig. 2, which is about a factor of two better than results from X-ray tube sources without a monochromatised Al $K\alpha_{1,2}$ source⁸. The splitting between the two peaks, and their relative heights (Fig. 2a, b), are in excellent agreement with earlier results³. Signal-background and signal-noise ratios are expected to be improved after a minor modification of the experimental set up. Three factors contribute to the observed linewidth of 0.42 eV at FWHM: the inherent width of the 4f photoline; the resolution of the energy analyser; and the resolution of the crystal monochromator (including the effects of slits and collimators). The energy resolution of the doublepass cylindrical mirror analyser is well known, and is 0.6% of the pass energy for the electrons. We chose a pass energy of 20 V, thus, the contribution to the resolution from the energy analyser is 0.12 eV. It is more

difficult to give an exact figure for the contribution from the crystal monochromator, because of the complexity of the slits and collimators, and because of the difficulty of estimating the source size. A consideration of all of the geometrical factors, however, suggests that it should lie somewhere between 0.25 eV and 0.35 eV. With the arbitrary assumption of Gaussian line shapes the inherent linewidth would then be 0.2–0.3 eV. It can be assumed, however, that the line shapes are neither pure Gaussian nor Lorentzian. All that we can therefore conclude at present is that the intrinsic linewidth is certainly less than 0.3 eV but probably 0.10–0.15 eV. We are not aware of any theoretical estimate of the different physical processes (for example, radiative and non-radiative Auger transitions) contributing to the linewidth. Published calculations of Auger transition rates do not extend out to the 4f shell⁹.

The calculated photon flux after the monochromator, using the SPEAR parameters already given, is 10^9 – 10^9 photons $s^{-1} cm^{-2}$ which is consistent with our flux measurement using an ionisation chamber. The useful flux on the sample is determined by the effective aperture of the energy analyser. Finally, we should comment on the counting rate which under the present running conditions of the storage ring and the electron spectrometer is 25 counts s^{-1} on the 4f peaks (Fig. 2). Obviously, this counting rate excludes the possibility of conveniently studying valence bands and core lines with considerably weaker intensity than the Au 4f levels. The intensity of 8,000 eV X rays will, however, increase about 50 times, after the upgrading of the SPEAR storage ring (planned for summer 1974), and thus the required data acquisition time will be considerably shortened.

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Electrically driven instability in elastic liquids

WE report here a striking phenomenon which we have observed in some elastic liquids. The phenomenon was discovered during the cleaning out of a glass vessel which contained a newly prepared solution of a polyisobutene of high molecular mass in a polyisobutene with a low molecular mass. The liquid was rather viscous, and so a good proportion of it remained on the walls of the

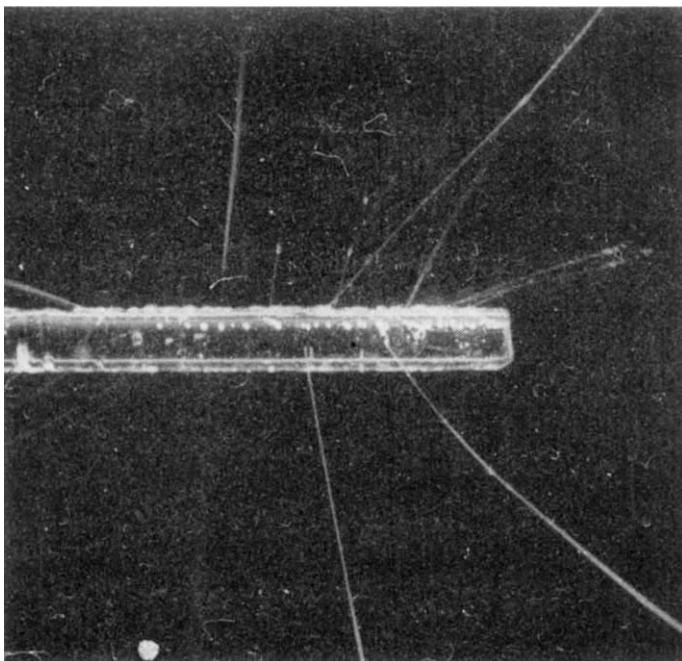


Fig. 1 The formation of fine jets of liquid after a polyisobutene solution has been torn away from a glass surface. The glass rod in the photograph has a diameter of 6 mm.

container. We decided to recover this by utilising the elastic properties of the solution and 'winding' it onto a glass rod. Using this method the liquid can be torn completely free from the walls of the container. Having thus removed some of the liquid from the container we found that the liquid on the rod spontaneously developed a number of fine streamers (Fig. 1). These emanated from irregularities on the surface of the liquid and their tips moved away from the rod at speeds of several cm s^{-1} .

Using an electroscope we established that the process of tearing the liquid from the walls of the container resulted in a separation of electrical charge. We also noticed that the streamers were readily attracted to nearby objects.

It has been known for many years¹ that the horizontal free surface of a liquid in a vertical electric field can be unstable, resulting in the formation of vertical jets of liquid if the field intensity is sufficiently large. We believe that the present phenomenon is an example of this instability. As the winding-on procedure produces irregularities in the free surface, the charge that is generated in the process of tearing the liquid from the container will not be uniformly distributed over the surface, thus giving rise to local field intensities near these irregularities much larger than the average. Presumably these 'local' fields can exceed the critical field intensity required for the described instability to occur, and a jet (or streamer) of liquid forms in exactly the same way as that described by Taylor¹. We have found that these streamers grow to great lengths (to many thousands of diameters) without breaking and can be so fine as to be quite difficult to see with the naked eye. This remarkable cohesion is most probably aided by the very good spinnability of many elastic liquids², which allows them to be drawn into extremely fine threads without breaking. (Taylor found in his experiments¹ that, if he used a conducting liquid, a steady uniform jet of liquid was formed when the field was sufficiently strong. If, however, the liquid was a poor conductor, such as transformer oil, the jet did not properly establish itself and took the form of a series of droplets. We repeated Taylor's experiment using the polyisobutene solution, which has an extremely low conductivity, and found that the instability resulted

in the formation of a steady uniform jet. We attribute this to the good spinnability of the non-Newtonian polyisobutene solution, as opposed to the Newtonian behaviour of transformer oil.)

We tried to reconstruct our observation by spreading a small quantity of the liquid (taken from the stock solution) on a glass plate and then 'tearing' the liquid from the plate by winding it on to a rod. The phenomenon was not observed immediately, but after the liquid had been left on the plate for a day in the open air, we found that the phenomenon could once more be produced easily. Further investigation indicated that the composition of the plate (whether glass, perspex, or a metal) did not affect the experiment noticeably, but that it would not work when the rod was a conductor.

At this stage we decided that the charge separation might occur in a similar manner to that in which charge arises when petrochemical mixtures flow through pipelines. In such cases it has been found that the formation of the charge depends on the presence of trace polar impurities³. In order to test this idea we placed some fresh liquid on a glass plate, put the plate in a desiccator for a week, and tried to observe the phenomenon. No streamers were found but after the liquid on the plate had been left exposed to the air for a few hours we were again able to produce the instability. It seems, therefore, that in our experiments a small amount of water from the air was necessary for the charge generation.

We believe that the extremely low electrical conductivity of polyisobutene solutions is an important feature of the experiment, as it is essential that the field is maintained while the streamers are forming, and this requires that the charge should not leak away too quickly. The phenomenon has also been observed with a solution of polystyrene in di-phthalate.

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Bordoni peak formation as a result of a martensitic transformation

THE indium-thallium alloys in the composition range 16–31 atom% Tl undergo a martensitic phase transformation from the higher temperature f.c.c. form to the lower temperature f.c.t. modification^{1,2}—a transformation which is well suited to ultrasonic studies. Here we report ultrasonic attenuation measurements, which have revealed the development of an internal friction peak which results from the passage of 27 atom% Tl alloy single crystals through the phase transition (which occurs at 127 ± 2 K for this composition³). The general behaviour of this peak strongly suggests a relaxation type of effect similar to that first observed by Bordoni in copper⁴. Such peaks are either absent or negligibly small in well annealed metals; but they are produced by plastic deformation and are considered to arise from thermally activated processes involving dislocations⁵. We can therefore expect that any dislocations which are formed as a result of the martensitic transition would give rise to such a peak, and that it could be detected

ultrasonically, provided that the investigated ranges of ultrasound frequency and temperature are appropriate to the activation energy involved.

Measurements were made on [110] oriented, single crystal samples of the alloy, of composition 27 atom% Tl, cut from a boule of about 4 cm³ which had grown in a horizontal zone furnace. Ultrasonic pulse-echo measurements were made at 14 MHz and 42 MHz with longitudinal waves and [100] polarised shear waves, respectively (Fig. 1). A large absorption peak occurs at the transition temperature T_c (refs 3 and 6). The peak temperature is independent of frequency, although it does differ somewhat between warming and cooling, as would be expected for this transition. The additional feature of the attenuation behaviour (shown in more detail in Fig. 2) is the peak which occurs at temperatures higher than T_c and which moved to higher temperatures when the driving frequency was raised. During each experiment the sample was taken through a cycle of cooling from room temperature to below T_c (thus producing a banded twin f.c.t. structure) and then warming back to 300 K to reverse the process and to re-establish the single crystal form; the temperature change was continuous, except at the end points. For the first few (≈ 5) cycles following the original spark cutting and planing of the crystal, the additional attenuation peak could be seen only during the warming part of the cycle and was not seen during cooling (Fig. 1). After a further few cycles, however, the peak became established during both warming and cooling (Fig. 1). Subsequent measurements over a long period of time showed that the peak height decreased gradually, until after about eight months it had vanished almost completely.

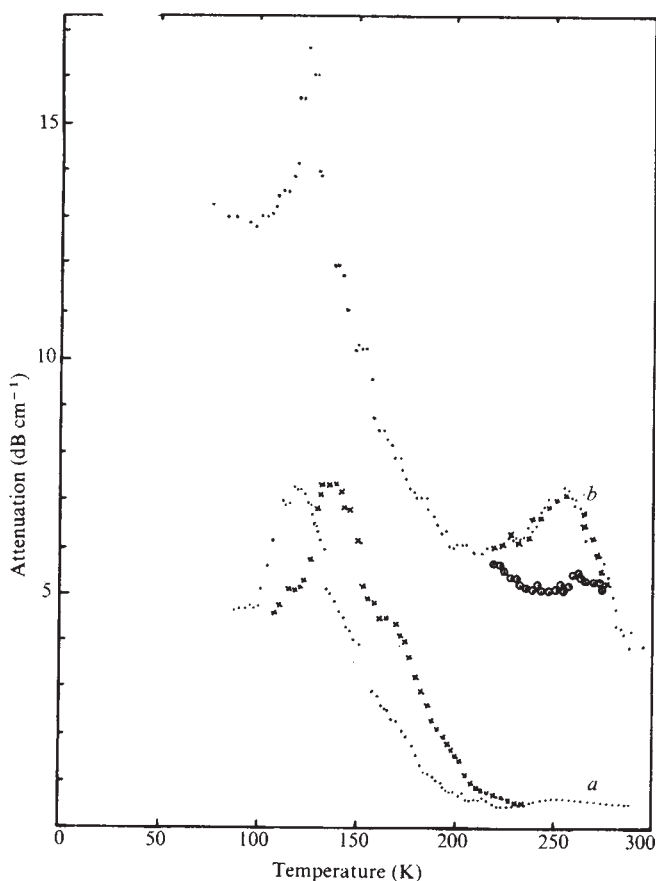


Fig. 1 The temperature dependence of the attenuation of ultrasonic waves propagated along the [110] direction in an In-27 atom% Tl alloy. *a*, 14 MHz curves (longitudinal waves); *b*, 42 MHz curves (shear waves, polarised [001]); ●, cooling; ×, warming; ⊙, initial cooling. The peaks at 127 K (on cooling) result from the phase transition, and the Bordoni peak appears at about 250 K.

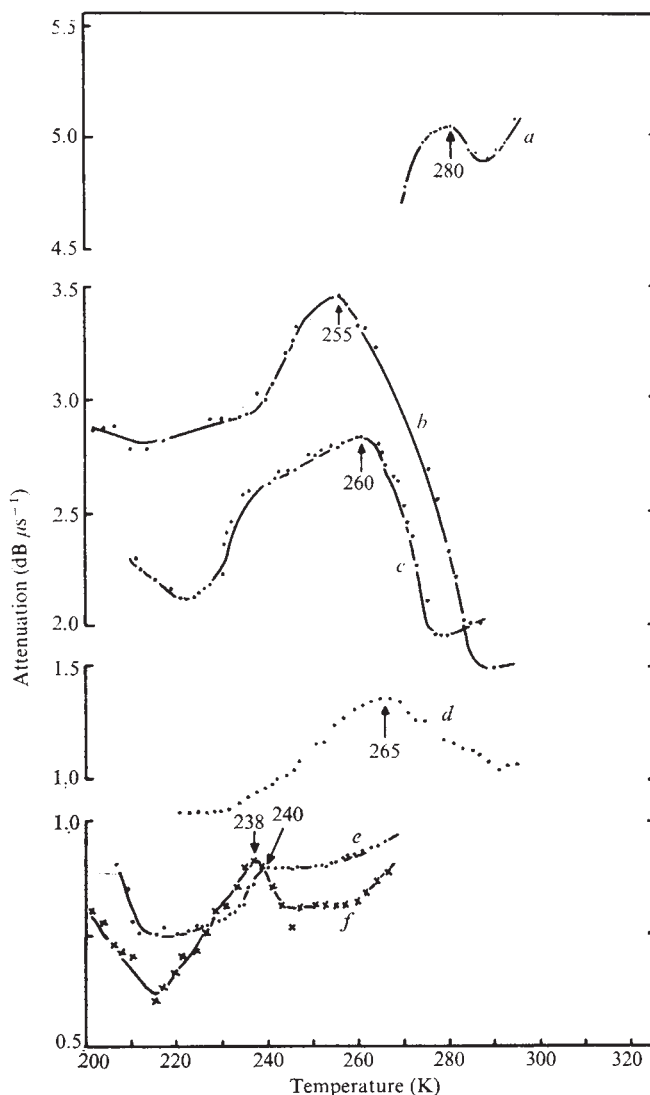


Fig. 2 Collected data of the Bordoni peaks found for ultrasonic waves propagated in the [110] direction in In-27 atom% Tl crystals. *a*, 70 MHz curve, shear wave (sample A); *b*, 42 MHz curve, shear wave (sample A); *c*, 42 MHz curve, longitudinal wave (sample B); *d*, 42 MHz curve, longitudinal wave (sample A); *e*, 14 MHz curve, longitudinal wave (sample B); *f*, 14 MHz curve, shear wave (sample A). These data were obtained during the period when the peaks could be seen on both warming and cooling, and before their amplitude had become too small to measure.

In general, the observed effects are characteristic of Bordoni peaks in f.c.c. metals. For example, Bordoni⁴ first found that the internal friction peak in copper was reduced by about 30% after annealing at 150° C for 10 h. Room temperature annealing of the Bordoni peak, of the kind observed here, is well known in aluminium^{7,8}. In indium-thallium alloys the peak temperature does not depend appreciably upon the number of passes through the transition. This corresponds with the usual finding: that the Bordoni peak position is not very sensitive to prestrain, annealing or impurities. The results in Fig. 2 can be used to test a relaxation process hypothesis. An activation energy, E , is defined, and the peak temperature T_m , and driving frequency, ν , can be related by an Arrhenius type of expression

$$\nu = \nu_0 \exp(-E/kT_m) \quad (1)$$

in which ν_0 is an attempt frequency. A straight line on an Arrhenius plot (Fig. 3) gives a reasonable fit to the present results. Several determinations were made of T_m at each frequency with different ultrasound wave polarisations, on two different [110] samples cut from the same boule, and the

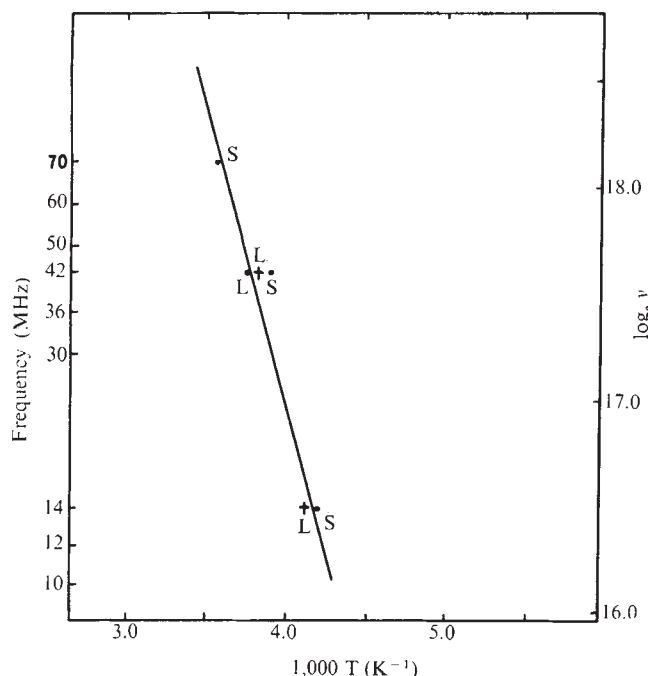


Fig. 3 Arrhenius plot to determine the activation energy and attempt frequency corresponding to the Bordoni peak, induced by passing through the transition. ●, Sample A; +, sample B; L, longitudinal waves; S, shear waves.

variations were found to be small. The activation energy, E , was found to be 0.24 ± 0.05 eV and the attempt frequency, ν_0 , $1.5 \pm 0.4 \times 10^{12}$ Hz. Applied to Bordoni peaks in f.c.c. metals in general this procedure gives activation energies of the order of 0.1 eV: for copper, E is 0.1–0.15 eV (with $\nu_0 = 10^{10}$ – 10^{13} Hz); for aluminium, E is 0.25 eV ($\nu_0 = 10^{14}$ Hz) (ref. 5). The peak parameters obtained for indium-thallium alloy studied here, fall into the same range. It is interesting that there is no significant difference, within the limits of experimental error, between the activation energies obtained for the longitudinal and the transverse modes. A difference can occur, however, when the resolved stress component differs on the slip plane corresponding to the polarisations of the longitudinal and transverse ultrasonic waves⁹.

The general features of the peak in the ultrasound attenuation in the alloy are consistent with those of Bordoni peaks. In other materials such peaks have been shown previously to result from dislocation motion, and to occur at high dislocation densities. The peaks in the 27atom% Ti alloy were formed after passage of the crystals through the martensitic transition, and back. If a dislocation damping mechanism is assumed, then not only is the frequency dependence of the peak temperature accounted for, but so is the observation that the peak appears initially only on warming. The strains involved during the transformation must produce a large number of dislocations which then interact with the ultrasound waves as the sample is warmed up, and anneal out when it is held at room temperature for several hours (300 K is approximately 0.7 of the melting temperature). Therefore, subsequent cooling does not result in the reappearance of the peak. But if after several cycles, the dislocations become pinned sufficiently for short period annealing to become ineffective in causing their removal, then the peak will appear in both the cooling and the warming parts of the cycle, as is observed. The attenuation peak results provide experimental evidence of the production of dislocations during the martensitic transformation.

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Determination of the gas constant by an acoustical method

A NEW value for the gas constant, R_0 , has been obtained from measurements of the velocity of sound, c , in argon at the temperature of the triple point of water. Velocity measurements were made using a low frequency variable-path acoustic interferometer, operating at a frequency of 5.6 kHz (ref. 1). Ninety eight independent measurements of sound velocity were made over a range of pressures from 0.3–2.0 atm. A quadratic least squares fit was made of c^2 against pressure, and the resulting acoustic isotherm was extrapolated to zero pressure to yield a value for c_0^2 , the velocity in the limit of low pressures. The gas constant was then obtained from the relationship

$$c_0^2 = \gamma R_0 T / M$$

where $\gamma = 5/3$, $T = 273.16$ K, $M = 39.9478$ g mol⁻¹ and c_0^2 was the experimentally determined value of $94,768.9 \pm 2.1$ m² s⁻². The uncertainty quoted for c_0^2 is the standard error on the intercept of the acoustic isotherm.

The value for the gas constant thus obtained is

$$R_0 = 8,315.59 \pm 0.18 \text{ J K}^{-1} \text{ kmol}^{-1}$$

The uncertainty quoted here is the standard error in the result, which stems from the standard error in c_0^2 arising from random errors in the measurements of sound velocity. It is equivalent to a standard error of 22 parts per million (p.p.m.) in R_0 .

The largest systematic uncertainty is that arising from the uncertainty in the isotopic composition of the argon used in the measurements, and is thought not to exceed 7 p.p.m. Errors in temperature measurement, acoustic frequency and the measurement of displacement in the interferometer are considered negligible, and errors resulting from chemical impurities in the argon are not thought to lead to a systematic uncertainty greater than 3 p.p.m. The major uncertainty in the result is thus that arising from random errors in the measurement of velocity.

Full details of the experimental method and results will be published.

This new value for R_0 is greater by $1.18 \text{ J K}^{-1} \text{ kmol}^{-1}$ (142 p.p.m.) than that based upon measurements of the density of oxygen and quoted as $8,314.41 \pm 0.26 \text{ J K}^{-1} \text{ kmol}^{-1}$ (ref. 2).

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Solution evaporation method for solid state ESCA studies

ALTHOUGH considerable effort in electron spectroscopy for chemical analysis (ESCA) has been channelled into the development of narrow line sources¹⁻⁴, there has been little attention to the problem of increasing ESCA resolution by decreasing the large linewidth contribution from involatile non-conducting solid samples. For such samples, most workers have spread a thin layer of solid on to sticky tape^{5,6}, or have used a metal mesh as host matrix⁷⁻⁹. Al has also been suggested as a sample backing by P. E. Larsen. These procedures generally provide widths approximately 1 eV broader than those from the gas phase¹, thin condensed phase¹⁰, or from conductor¹ spectra. The procedures normally involve the use of a few mg of compound, even though ESCA should be sensitive to $\sim 10^{-8}$ g of a material^{2,11}.

We describe here a simple technique for evaporating very dilute solutions of compounds on to acid-etched Al plates which, first, usually appreciably decreases the linewidths of non-conducting samples; second, enables good spectra to be obtained using μg amounts of compound; and third, minimises charging effects and calibration problems.

All spectra were recorded using an ESCA-36 McPherson Instrument and Mg K α radiation. To avoid decomposition of samples, low X-ray power (15 mA $\times$ 8 kV) was used for as short a period as possible. Spectra were calibrated using the Au evaporation technique^{12,13}, and an argon ion gun (10 keV argon ions) was used to etch a Sn metal foil *in situ* in order to obtain a spectrum of pure Sn metal. Besides the energy levels of direct interest in this study

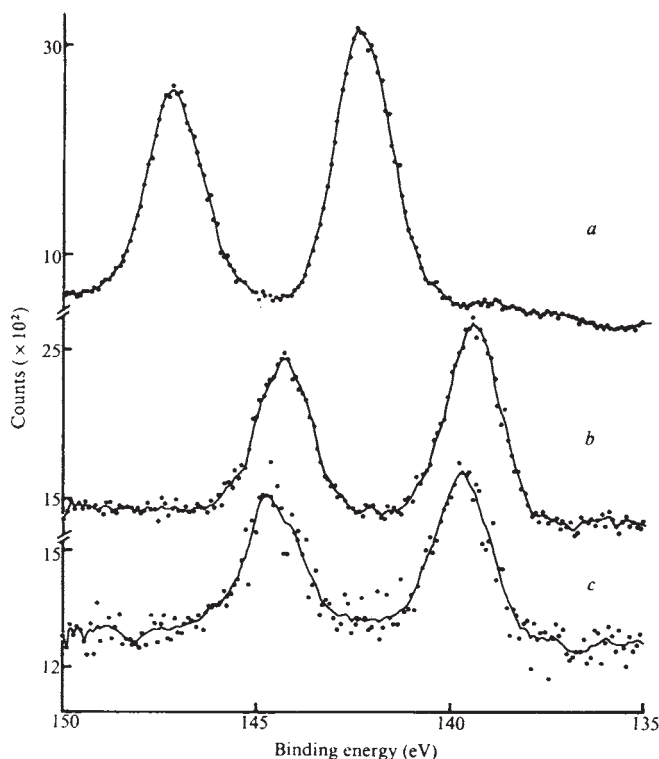


Fig. 1 Pb 4f spectra of $\text{Pb}(\text{NO}_3)_2$ taken using a Mg anode and an X-ray power of 120 W. The binding energies are uncorrected for charging effects. See Table 1 for corrected values. The lines drawn through the spectra are seven point smooths. *a*, 6.1 mg of solid $\text{Pb}(\text{NO}_3)_2$ spread evenly onto an acid etched Al plate; time of accumulation = 15 min. *b*, 3.8 μg of $\text{Pb}(\text{NO}_3)_2$ from 10 μl of a $1.6 \times 10^{-3}\text{M}$ $\text{Pb}(\text{NO}_3)_2$ solution in H_2O ; time of accumulation = 75 min. *c*, 0.4 μg of $\text{Pb}(\text{NO}_3)_2$ from 10 μl of a $1.16 \times 10^{-4}\text{M}$ $\text{Pb}(\text{NO}_3)_2$ solution in H_2O ; time of accumulation = 75 min.

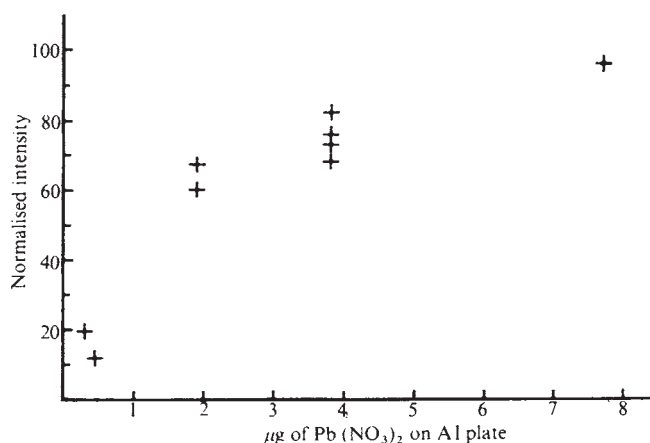


Fig. 2 Normalised intensity of the Pb 4f_{7/2} line against amount of $\text{Pb}(\text{NO}_3)_2$ (μg) on the Al plate.

(Pb 4f, and Sn 3d), spectra of energy levels of other elements (such as C 1s, N 1s and O 1s) in the compounds were always recorded.

Alcan AA 3003 aluminium plates (2 cm $\times$ 1 cm $\times$ 0.06 cm) were lightly etched for 2–3 min in 3 M HCl until they were readily wettable with H_2O . Purer Al (Fischer 99.9% purity) was also tried with similar results. More extensive etching or roughening with fine emery paper invariably leads to the poorer results. Pt foil further reduced charging effects but did not wet as well. The etched Al plates were washed with H_2O and acetone and dried in an oven at 70° C.

Generally, solutions of between 10^{-2} M and 10^{-4} M of a given compound were prepared and 3–20 μl pipetted on to the Al plate. Volatile solvents such as benzene readily spread over the plate. Because of the high surface tension of H_2O , the end of the micropipette was used to spread aqueous solutions over the plate; if the plate was sufficiently etched, the water would wet the entire plate. The solvent was then allowed to evaporate in air.

The use of such small amounts of samples is an aid to minimising vacuum problems encountered with slightly volatile or unstable compounds.

An adequate spectrum of 0.4 μg of $\text{Pb}(\text{NO}_3)_2$ on a 2 cm² plate can be obtained in 75 min, using low X-ray power, and the linewidths (1.60 eV) are narrower than those (1.70 eV) for the 6 mg of $\text{Pb}(\text{NO}_3)_2$ solid (Fig. 1c). It is interesting to compare our results with those obtained by Hercules *et al.*¹¹ using complexing surface groups. We obtained the spectrum on 0.4 μg of compound, whereas they used 2,400 scans (for an unspecified length of time) to obtain a spectrum on ~ 2 μg of Pb.

Considering the area of the Al plates (2.0 cm²), assuming that the area taken up on the surface by a $\text{Pb}(\text{NO}_3)_2$ molecule is $\approx 20 \text{ \AA}^2$ and that there is an even distribution of $\text{Pb}(\text{NO}_3)_2$ molecules on the surface, 1 μg of $\text{Pb}(\text{NO}_3)_2$ corresponds to 1.8 monolayers. Thus, the spectrum in Fig. 1c corresponds to that for about one monolayer of $\text{Pb}(\text{NO}_3)_2$. This suggests that considerably smaller amounts of $\text{Pb}(\text{NO}_3)_2$ could be detected at higher X-ray powers and longer scan times.

Furthermore, the reproducibility of the results (Fig. 2) is surprisingly good considering the potential difficulties in obtaining an even distribution of molecules on a surface. It is also interesting that the intensity begins to level off after a few μg of compound, although we have found that the normalised intensity continues to increase slowly with increasing amounts of $\text{Pb}(\text{NO}_3)_2$ on the plate. The reproducibility, combined with the narrower linewidths and the levelling off of the intensities, strongly indicates a reasonably even distribution of molecules on the surface.

Table 1 Pb 4f binding energies

No. of spectra	Amount of Pb (NO ₃) ₂ on plate (μg)	Pb 4f uncorrected (± 0.1 eV)	Pb 4f uncorrected with Au (± 0.1 eV)	Au 4f _{7/2} (± 0.1 eV)	Pb 4f corrected (± 0.2 eV)*
2	1.9	144.3	144.3	84.6	143.7
4	3.8	139.5	139.3		138.7
		144.5	144.5	84.8	143.7
1	7.6	139.6	139.6		138.8
		144.4	not obtained		
2	76	139.6			
		144.5	144.1	84.3	143.7
3	6,100	139.6	139.2		138.8
	solid	147.2	146.8	87.0	143.8
	none	142.4	141.9		138.9
				84.2	

* Au 4f_{7/2} = 84.0 eV.

Figure 2 indicates that with further improvement of the technique, there could be two very important uses both in ESCA and Auger spectroscopy: calibration curves for quantitative analysis, and escape depth studies.

The results (Fig. 1 and Table 1) also indicate that charging effects are minimised in the solution evaporated samples, relative to the solid spectrum, although the corrected binding energies are the same within the error for all samples. Pb(NO₃)₂ from solution evaporation is still about 0.5 eV compared to 139.6 eV for a solution evaporated sample, whereas the corrected Pb 4f_{7/2} peak is at 138.9 eV and 138.8 eV, respectively. The Au calibrations show that the Pb (NO₃)₂ from solution evaporation is still about 0.5 eV charged relative to Au on the Al plate. Thus, the Au 4f_{7/2} line (84.0 eV for Au metal) is at 84.2 eV on the etched Al plate, but is at 84.6±0.2 on the Al plates with evaporated Pb(NO₃)₂. Au linewidths of 1.40±0.05 eV were obtained for all Au evaporations.

It is apparent that the solution evaporation technique is ideally suited to internal calibration as used in other forms of spectroscopy, and in ESCA spectra of volatile solids in which a compound such as perfluorocyclohexane

(C₆F₁₂) is cocondensed with the compound of interest¹⁴.

For many molecular Sn(IV) compounds, the solution evaporation method leads to significant decreases in linewidths relative to spectra taken in the conventional manner, especially when large charging shifts are present. The minimum Sn 3d linewidths for a conductor (Sn metal) obtainable on our equipment are 1.05±0.05 eV (Fig. 3a) whereas that for a molecular non-conductor (Ph₄Sn) is 1.20±0.05 eV. In contrast, typical 3d linewidths on Ph₄Sn solid are 1.50±0.05 eV. Sn 3d linewidths for a number of other Sn compounds are 1.30±0.05 eV, but others such as Me₂Sn (acac)₂ (acac=anion of acetylacetone) give markedly larger linewidths of 1.65±0.05 eV. Solid samples have larger linewidths probably because of differential charging⁹. For some molecular Sn compounds, narrow linewidths can also be obtained by using very thin solids spread on to the etched Al plates.

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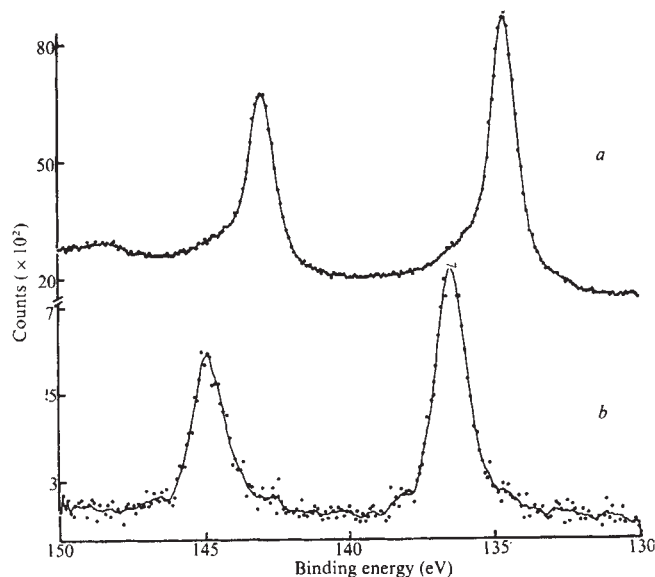


Fig. 3 Sn 3d spectra taken at an X-ray power of 120 W. The lines are seven point smooths. a, Sn metal, produced by an 8 min argon ion etch of Sn foil. The small shoulders on the left hand sides of the peaks are because of residual tin oxides. Time of accumulation=3.7 min. b, 12 μg of (C₆H₅)₂Sn from 3 μl of 9.3 × 10⁻³M benzene solution of (C₆H₅)₂Sn. Time of accumulation=15 min.

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BIOLOGICAL SCIENCES

Actinomycin D and RNA transport

ACTINOMYCIN D, a potent inhibitor of RNA chain propagation of DNA-directed RNA transcription¹⁻³, has been widely used for studies of turnover and transport of RNA in a variety of cell systems, to try to demonstrate that nuclear RNA labelled before the addition of actinomycin D is transported to the cytoplasm. It has been found that most labelled RNA remains in the nucleus⁴⁻⁸. It has been shown that the decrease in radioactivity of the heterogeneous nuclear RNA in HeLa cells is not accompanied by transfer of appreciable material to the cytoplasm⁹. Results of such experiments have even indicated that no transfer of rapidly labelled RNA from nucleus to cytoplasm occurs⁹. Recent studies on the effect of actinomycin D have raised doubts as to the validity of conclusions based on experiments including it. A prolonged half-life of mRNA due to actinomycin D has been observed in liver cells¹⁰, and the decay time of mRNA in HeLa cells has been found to be considerably shortened by treatment with this antibiotic¹¹. A non-physiological breakdown of rapidly labelled RNA by actinomycin D in primary chick fibroblasts has also been reported¹².

In studies of RNA transfer from nucleus to cytoplasm by chase experiments with actinomycin D, the length of the labelling period before inhibition is very important. Actinomycin D, being a template inhibitor, binds to DNA, and thereby efficiently blocks elongation of nascent RNA molecules. The use of too short pulse labelling before administration of the drug may imply that, at the onset of inhibition, most of the radioactivity is confined to nascent RNA chains that cannot be released from the chromosomal sites during the ensuing chase with actinomycin D. Here I describe experiments supporting this idea. Transport of labelled chromosomal high molecular weight RNA to the cytoplasm after actinomycin D chase can be demonstrated if the period of prelabelling is sufficiently long (more than 25 min), and if labelled RNA has appeared in the nuclear sap before the addition of inhibitor.

Salivary glands were isolated from fourth instar larvae of the dipteran *Chironomus tentans*¹³. Four animals were used in each experiment. Four salivary glands were explanted into 50 μ l of modified Cannons medium¹⁴⁻¹⁵ supplied with tritiated cytidine and tritiated uridine (Amersham) at specific activities of 25–30 Ci mmol⁻¹. After prelabelling for 25 or 45 min, actinomycin D (Calbiochem, Los Angeles) was added. The sister glands were used as control glands and the incubations were carried out at 18° C. Fixation, microdissection, extraction of RNA and electrophoresis in 1% agarose were performed as described elsewhere¹⁶⁻¹⁸. After the electrophoretic run, the gel slabs were treated with cold TCA to aid the removal of non-specific label¹⁹. The analyses of nuclear and cytoplasmic RNA from glands incubated with actinomycin D showed that labelling was inhibited by more than 98% (ref. 16). After 90 min of normal labelling with tritiated nucleosides, the chromosomal RNA acquired nearly maximal labelling, and a large amount of label appeared in the cytoplasm. A total length (pulse + chase) of 90 min was therefore considered as a suitable period for incubation.

The electrophoretic analyses of chromosomal RNA (Fig. 1a), nuclear sap RNA (Fig. 1b) and cytoplasmic RNA (Fig. 1c) after 25 min of labelling and after 25 min of labelling plus 65 min of synthesis inhibition with actinomycin D are presented in Fig. 1. The profile of chromosomal RNA after 25 min of incorporation displays the usual bimodal distribution

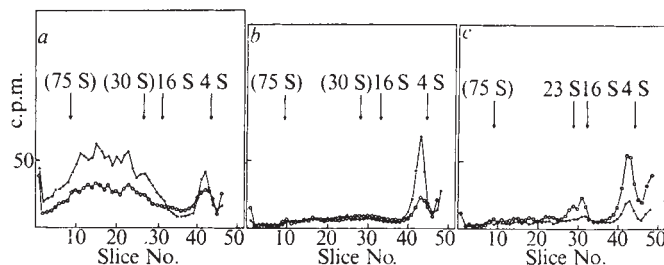


Fig. 1 Electrophoretic separations of, *a*, chromosomal RNA, *b*, nuclear sap RNA and, *c*, cytoplasmic RNA after 25 min of labelling and after 25 min labelling + 65 min of synthesis inhibition. Four glands from four different animals were placed in an incubation droplet of 50 μ l containing 100 μ Ci tritiated cytidine and 100 μ Ci tritiated uridine, and their sister glands were placed as controls in another 50 μ l of the same medium. The glands were incubated at 18° C for 25 min. Next, actinomycin D was introduced to one of the droplets at a concentration of 10 μ g ml⁻¹ and the glands were incubated for another 65 min, while the incubation of the control glands was stopped. After incubation the glands were fixed, and the cell components from five cells per gland were isolated by micro-dissection. The labelled RNA from each sample was released by pronase-sodium dodecyl sulphate treatment, and the electrophoresis was carried out in 1% agarose gel. To remove actinomycin D and RNase-resistant radioactivity the gels were treated with cold TCA¹⁹. At the end of the run the whole gel slab was placed in a 250 ml beaker containing 5% TCA at 4° C, and washed for 60 min. The treatment was repeated in a new volume of TCA and the gel was finally washed twice for 40 min in 1 l of distilled water at 4° C. Before radioactivity measurement, the gel was sliced and the slices were transferred to Packard counting vials, each containing 10 ml of toluene scintillator (1 l of which contains 30 ml toluene (Packard), 5.5 g of Permablend (Packard) and 20 ml methoxyethanol), incubated for 3 h at 60° C and finally counted in a Packard liquid scintillation spectrometer (Model 3380). *E. coli* RNA was used as marker (23S, 16S and 4S). The position of 75S and 30S were determined in parallel analyses of BR 2 RNA nucleolar RNA respectively. ●, Normal cells; ○, cells treated with actinomycin D.

with a peak in the 4S range and with label heterogeneously distributed in the 16–100S range. The subsequent treatment with actinomycin D for 65 min reduced the labelling of chromosomal RNA to 50–60% of the control values rather uniformly in the whole spectrum. The electrophoretic pattern of 25 min-labelled nuclear sap and cytoplasmic RNA shows only trace amounts of label in the high molecular weight ranges, but there is a rather distinct peak in the 4S region. During the ensuing 65 min of actinomycin D chase, no significant quantity of labelled heterogeneous RNA was released from the chromosomes into the nuclear sap. It is, of course, not possible to exclude that a release of heterogeneous RNA to nuclear sap may take place, but the product must be degraded instantaneously after its detachment. The radioactivity profile of cytoplasmic RNA displays an enhanced labelling in the 4S region as an expression of 4S RNA transport from the nucleus to the cytoplasm²¹. There is also a slightly increased radioactivity level in the position of the rRNA components, but there is no significant increase in labelling in the range above 28S. Thus, in spite of the fact that a considerable reduction of labelled chromosomal heterogeneous RNA takes place during actinomycin D chase, there is no corresponding increase of label either in nuclear sap or in the cytoplasm. Practically no rapidly labelled high molecular weight RNA moves out from the nucleus to the cytoplasm if labelling for 25 min is combined with 65 min actinomycin D chase.

It is yet not understood whether nascent RNA chains blocked by actinomycin D are detached and then broken down or if they are broken down without leaving the transcription sites. Electron micrographs of nucleolar DNA, containing nascent preribosomal RNA in 'Christmas tree' formation, show a random loss of incomplete RNA molecules during actinomycin D treatment irrespective of the

length of molecules (Mr F. Trendelenburg and W. W. Franke, unpublished.) A random and nonphysiological release of unfinished RNA chains may be the interpretation of the electrophoretic analyses of heterogeneous chromosomal RNA showing a parallel decay of labelling in the whole spectrum after actinomycin D chase. A labelling period of 25 min before the addition of the drug is sufficiently long, however, for completion of a substantial amount of 4S RNA, and during the subsequent actinomycin D treatment, transport of labelled 4S RNA from nucleus to cytoplasm can be demonstrated.

The electrophoretic analyses of labelled RNA from chromosomes (Fig. 2a), nuclear sap (Fig. 2b) and cytoplasm (Fig. 2c) after 45 min of labelling and after 45 min of labelling plus 45 min of actinomycin D chase are shown in Fig. 2. The pattern of labelled chromosomal RNA after 45 min of labelling shows a bimodal distribution similar to that after 25 min of incubation (Fig. 2a). Whereas the labelling of chromosomal RNA during 45 min of actinomycin D chase decayed 30–35%, the relative distribution of the heterogeneous RNA remained roughly the same as in the control profile. The emergence of a distinct radioactivity peak around the 30S region in the drug-treated glands can be explained by accumulation of preribosomal RNA of nucleolar origin on the chromosomes¹⁵. This appearance of preribosomal 30S RNA on the chromosomes seems to be potentiated by treatment with actinomycin D. The electrophoretic pattern of nuclear sap RNA after 45 min of labelling contains, besides a distinct 4S RNA peak, label distributed rather heterogeneously in the 16–100S range, with a small peak in the 75S region (Fig. 2b). The material distributed between 16–30S is likely to be of ribosomal origin¹⁵. When a normal 45 min pulse is followed by synthesis inhibition for 45 min, the radioactivity profile of nuclear sap RNA exhibits a decrease (30–35%) compared with the normal profile, except for rRNA in the 16–30S range, which is slightly increased. The reduction of label is most pronounced in the 4S and 35–40S regions. The electrophoretic analysis of labelled cytoplasmic RNA shows distinct peaks in the 4S and 18S regions and a small one in the 28S region (Fig. 2c). There is also a minor but significant peak in the 75S region¹⁷. 75S RNA as well as rRNA appears in the cytoplasm somewhat earlier than has been previously described¹⁷. After 45 min of actinomycin D chase the

electrophoretic pattern displays a generally increased labelling in the whole spectrum, although the increase is less in the highest molecular weight range. Distinct peaks can be seen in the 4S region and in the position of the ribosomal 18S and 28S RNA components. An additional rather discrete radioactivity component emerges in the 35–40S range during actinomycin D chase. It can be seen that the reduction of labelled RNA during actinomycin D chase is largest in this range of the profile of nuclear sap RNA. This cytoplasmic 35–40S RNA has characteristic labelling kinetics, and thus this peak is not an artefact due to actinomycin D treatment. A more extensive description of this RNA fraction will be presented elsewhere.

In spite of the lack of release of prelabelled heterogeneous RNA from the chromosomes during synthesis inhibition with actinomycin D (Fig. 1), radioactivity is increased in the high molecular weight range of cytoplasmic RNA (Fig. 2). This means that prelabelled nonribosomal RNA is being transported from the nuclear sap. In fact, more than 80% of the increase in radioactivity associated with the nonribosomal cytoplasmic RNA in the 16–100S range can be accounted for by the decrease of label in the same range of the nuclear sap profile. Only 30–35% of the prelabelled nuclear sap RNA, however, disappears during 45 min of actinomycin D chase.

I have found that after 25 min incorporation of radioactive precursors into *Chironomus tentans* salivary glands, the chromosomal RNA is labelled, but no appreciable amount of label appears in the nuclear sap and cytoplasm except in the 4S region. During a subsequent actinomycin D chase for 65 min the labelling of heterogeneous chromosomal RNA decreases by 40–50% but the decrease is not accompanied by an increase of label in the nuclear sap and cytoplasm except in the 4S range. A labelling period of 45 min is sufficiently long to allow a substantial amount of labelled heterogeneous chromosomal RNA to enter the nuclear sap and cytoplasm. If a labelling period of 45 min is followed by a 45 min actinomycin D chase, an export of nonribosomal high molecular weight RNA from the nucleus to the cytoplasm can be demonstrated.

One could explain the findings of the present work as follows; first, the labelled heterogeneous chromosomal RNA consists mainly of nascent chains or of nascent and finished RNA chains, all bound to the DNA template and, second, the appearance of finished RNA molecules (processed or unprocessed) in nuclear sap before the addition of actinomycin D is a prerequisite for transport of labelled RNA from nucleus to cytoplasm. Although some qualitative aspects of RNA transfer from the nucleus to the cytoplasm can be studied by the pulse-chase technique including actinomycin D, it is definitely not a method of choice for examining turnover rate, processing of rapidly labelled RNA and for quantitation of heterogeneous RNA transfer from nucleus to cytoplasm. This is because actinomycin D interferes with the completion and release of heterogeneous chromosomal RNA, and the contribution of nonreleasable label to the total labelled heterogeneous nuclear RNA cannot be determined in most cell systems.

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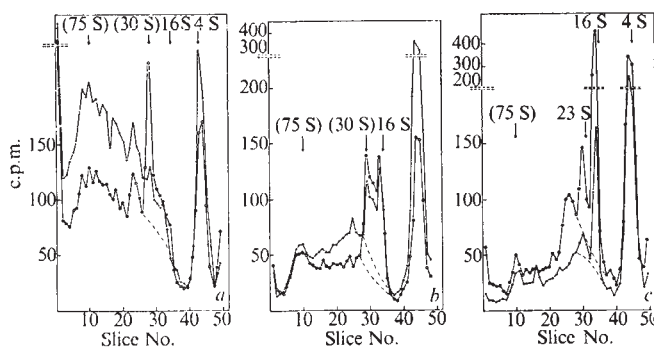


Fig. 2 Electrophoretic separation of, a, chromosomal RNA, b, nuclear sap RNA and, c, cytoplasmic RNA after 45 min labelling and after 45 min labelling + 45 min of synthesis inhibition. Four glands from four different animals were placed in 50 μ l of incubation medium containing 100 μ Ci tritiated cytidine and 100 μ Ci tritiated uridine, and their sister glands were placed in another 50 μ l of the same medium. The glands were incubated at 18° C for 45 min. Next, actinomycin D was introduced to one of the droplets at a concentration of 10 μ g ml⁻¹ and the glands were incubated for another 45 min, while the incubation of control glands was interrupted. Cell components from five cells per gland were dissected. ●, Normal cells; ○, cells treated with actinomycin D.

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Defective DNA repair in Fanconi's anaemia

FIBROBLASTS from patients with Fanconi's anaemia (FA) show increased susceptibility to transformation *in vitro* by SV40 virus¹, as well as spontaneous chromosome breakage². Chromosomal instability has also been observed in cultured peripheral blood lymphocytes and in bone marrow cells³. More recently, Sasaki and Tonomura⁴ demonstrated an increased susceptibility of lymphocytes from patients with FA to chromosome breakage by DNA cross linking agents. These results were interpreted as an indication that FA cells are defective in some DNA repair mechanism.

The studies we report here demonstrate that DNA repair by fibroblasts from a patient with FA is defective and suggest that the abnormality is due to a defect in the excision of DNA lesions. This is probably the result of a deficiency of a specific exonuclease.

The first step in the removal of ultraviolet-induced thymidine dimers or any other DNA damage which does not directly produce breaks in the DNA is the production of a single-strand scission by an endonuclease⁵. This can be demonstrated in normal cells by observing the shift in sedimentation of DNA, 90 min after irradiation with ultraviolet light, from the normal distribution to one in which the small DNA fragments sediment at a much slower rate (Fig. 1a). Figure 1b shows the results of incubating the cells for 10 h after they have been irradiated. A fraction of the DNA shifted back toward the normal sedimentation peak, suggesting that repair was taking place.

There was also a reduction in the S value of DNA in FA cells as a result of ultraviolet irradiation. Figure 1a shows that 90 min after FA cells were subjected to ultraviolet irradiation of 250 ergs mm⁻², the major peak disappeared and the DNA sedimented at the lower sedimentation rate characteristic of the light DNA occurring in FA cells in the absence of ultraviolet irradiation. Incubation of FA cells for 10 h after ultraviolet irradiation (Fig. 1b) did not result in any shift back towards the normal sedimentation pattern. These results suggest that the cells can produce single strand breaks after ultraviolet irradiation,

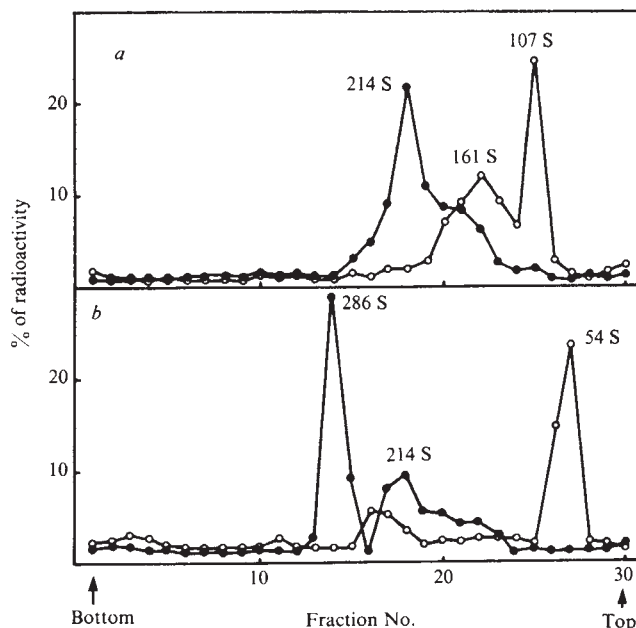


Fig. 1 Alkaline sucrose gradient sedimentation of labelled DNA from normal (●) and Fanconi's anaemia (○) fibroblasts (American Type Culture Collection No. HG261) after ultraviolet irradiation: *a*, 90 min after 250 ergs mm⁻²; *b*, 10 h after 250 ergs mm⁻². Cells were grown as a monolayer in minimal Eagle's medium with Earle's balanced salt solution supplemented with 10% foetal calf serum (inactivated), kanamycin (100 μg ml⁻¹), and non-essential amino acids. Two days after seeding the medium was replaced by medium containing ³H-thymidine (0.2 μCi ml⁻¹, specific activity 50 Ci mmol⁻¹, Amersham/Searle), and the cultures were grown for 18-22 h at 37° C in an atmosphere of 5% CO₂-95% air. The monolayer cells were washed three times with calcium and magnesium-free phosphate-buffered saline (PBS) and scraped or trypsinised from the flasks. The cell suspension was washed again and diluted to 5 × 10⁵ cells per ml with PBS. The sucrose solution was made with nuclease-free crystal sucrose (Schwarz/Mann) in double distilled water containing 0.9 M NaCl, N NaOH and 1 mM EDTA-disodium, pH 12.2. Linear sucrose gradients in polyallomer centrifuge tubes (36 ml) with a concentration of 5% sucrose at the top and 20% at the bottom were generated by a Beckman density gradient former. At the top of each gradient 0.3 ml of 0.5 N NaOH were overlaid and 0.3 ml of cell suspension (5 × 10⁵ cells per ml labelled with ³H-thymidine in PBS) was gently pipetted in. The tubes were kept for 2 h at room temperature and at 4° C for 16 h. The gradients were centrifuged at 23,000 r.p.m. for 2 h at 10° C in an SW 27 rotor of a Spinco Model L3-50 (Beckman) ultracentrifuge. Fractionation of the gradients was performed with a hypodermic needle pierced through the bottom of the tube and fractions were collected with a peristaltic pump through an automated fraction collector. Each fraction was precipitated by addition of 5% trichloroacetic acid, collected on an MF Millipore filter. Radioactivity on the dried filters was determined by transfer to 10 ml of a toluene based scintillation cocktail which was counted in a Beckman LS 250 liquid scintillation spectrometer.

tion, indicating the presence of an endonuclease, but cannot complete the repair process.

After cells have been irradiated with ultraviolet light and produce a single strand break (nick) near the induced pyrimidine dimers, the next step in the repair process involves the unwinding of the two strands in the neighbourhood of the nick and the polymerisation of a new strand of DNA complementary to the intact member of the DNA double helix⁵. This polymerisation can be demonstrated by unscheduled DNA synthesis detected autoradiographically by the incorporation of ³H-thymidine into the cellular DNA or, more specifically, by repair replication as assayed by Cleaver⁶. Unscheduled DNA synthesis in FA cells is comparable with that which occurs in normal cells (Fig. 2).

The incorporation of ^3H -thymidine, as indicated by the number of grains over the nuclei of the irradiated cells, increased as a function of the dose of irradiation and there was no distinguishable difference between the relative number of grains in FA cells and normal cells.

Unscheduled DNA synthesis was induced by treating FA cells with 4-nitroquinoline-N-oxide (4NQO), a carcinogen known to induce unscheduled DNA synthesis and replication repair synthesis in human cells (our unpublished studies). This agent induced no unscheduled DNA synthesis in the cells of patients with xeroderma pigmentosum^{7,8}, suggesting that it does not produce single-strand breaks directly. The fact that it induced unscheduled synthesis in FA cells suggests that they can make single-strand scissions after treatment with 4NQO as well as after irradiation with ultraviolet light.

To complete the repair of ultraviolet-induced DNA damage, it is necessary for the damaged strand to be excised so that the newly synthesised strand can be linked by phosphodiester bonds to the end of the remaining portion of the damaged strand. This is generally considered to be the function of a specific exonuclease. Exonucleases which seem to be specific for ultraviolet-induced damage have been isolated from cells⁹. The excision of thymidine dimers can be demonstrated by measuring the dimer content of irradiated DNA immediately after irradiation and again after irradiated cells have been incubated for a repair period. After the irradiation the number of dimers detectable in the DNA diminishes⁵. Figure 3 shows the results of an experiment in which normal cells were exposed to various doses of ultraviolet light and the number of dimers was determined in the DNA immediately and after a 24 h incubation. The number of thymidine dimers was a function of the ultraviolet dose over the range examined and after 24 h a large fraction of the dimers had been removed from the DNA of the normal cells.

When FA cells were subjected to the same doses of ultraviolet irradiation, the number of dimers measured immediately was essentially the same as seen in normal cells (Fig. 3). During a 24 h postirradiation period, however, FA cells could remove only a small fraction of the induced dimers. This difference was not apparent at ultraviolet doses below 150 ergs mm^{-2} , possibly because the FA cells could remove during the 24 h incubation,

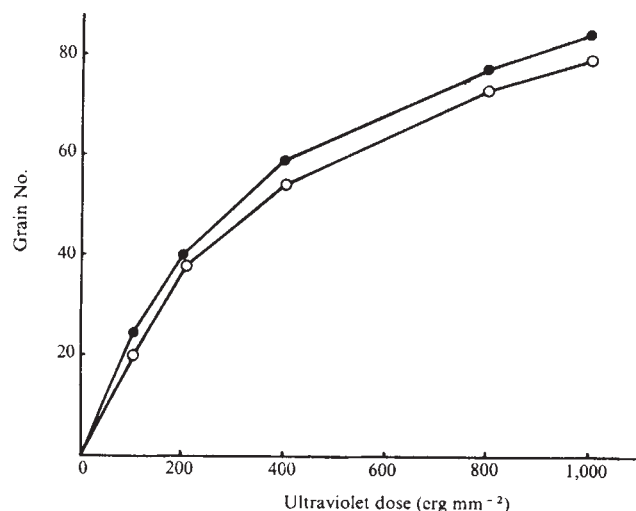


Fig. 2 Effect of ultraviolet irradiation on grain count of normal fibroblasts (●) and FA fibroblasts (○). Cells on coverslips were rinsed with PBS and pulsed with ^3H -thymidine ($2 \mu\text{Ci ml}^{-1}$, 50 Ci mmol^{-1}) for 1 h to identify cells in S phase. After ultraviolet irradiation at various doses, cells were exposed to ^3H -thymidine again for 2 h. Autoradiographs were prepared and the average grain count over lightly labelled cells was determined after a 2-week exposure.

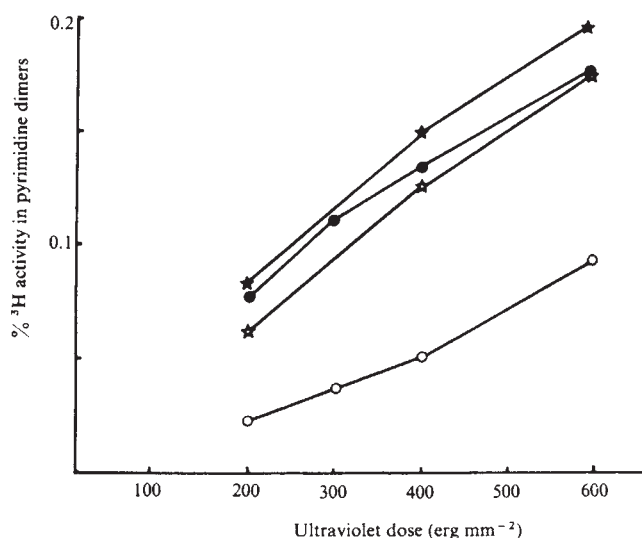


Fig. 3 Comparison of excision of thymine-containing pyrimidine dimers from ultraviolet-irradiated, ^3H -thymidine-labelled normal human fibroblasts and fibroblasts from a patient with FA: normal human fibroblasts immediately after irradiation (●) and 24 h after irradiation (○); FA cells immediately after irradiation (★) and 24 h after irradiation (☆). Cells growing in a glass Petri dish were labelled with ^3H -thymidine ($5 \mu\text{Ci ml}^{-1}$, 50 Ci mmol^{-1}). Cultures were then rinsed with PBS and irradiated with ultraviolet light at a dose rate of $10 \text{ ergs mm}^{-2} \text{ s}^{-1}$. After irradiation some cultures were fixed immediately with perchloric acid in the cold. Others were rinsed with PBS and allowed to grow for 24 h and then fixed. 5×10^6 cells were scraped into a Pyrex tube ($7.5 \times 100 \text{ mm}$) and precipitated with 5% trichloroacetic acid. The precipitate was washed, dried and hydrolysed in 0.2 ml of 98% formic acid by heating at 175°C for 2 h. The hydrolysates were spotted on a sheet of Whatman No. 1 paper for two-dimensional chromatography and the percentage of radioactivity was measured. This method cannot distinguish the difference between normal and FA with an ultraviolet dose below 150 ergs mm^{-2} . Either FA cells demonstrate normal excision at a low dose of ultraviolet light or the method is not sufficiently sensitive to detect it.

the relatively small number of dimers produced by lower doses of ultraviolet light. This indicates that although the FA cells could produce the endonucleolytic scission as the first step of the repair process and could polymerise nucleotides to repair or replace the damaged strand of DNA, they were deficient in the ability to remove the strand of DNA bearing the abnormal thymine dimers.

We have demonstrated that FA cells possess most of the functions required for the repair of ultraviolet-induced DNA damage. They can produce single strand scissions in response to pyrimidine dimer induction and they can polymerise nucleotides into a new complementary strand to replace that damaged portion of the DNA molecule. However, they seem to be deficient in an exonuclease function which removes the damaged strand of DNA after the endonucleolytic strand scission has been made. It is interesting that the FA cells studied seem capable of rejoining X-ray-induced strand breaks, suggesting that ligase activity is present (our unpublished data).

Regan *et al.* have stated that FA cells can normally excise ultraviolet-induced pyrimidine dimers¹⁰. The reason for the discrepancy between their results and ours is not clear, but may relate to their use of lower doses of ultraviolet irradiation. Our studies have demonstrated that a dose of at least 150 ergs mm^{-2} is necessary to demonstrate a difference between normal and FA cells. Thus, although FA cells do not seem to be as sensitive to ultraviolet light as xeroderma pigmentosum cells, they are more sensitive than normal cells. In fact, our results

indicate that exonuclease activity is not completely lacking. This may also explain the lack of ultraviolet-induced skin cancer in these patients.

Another possibility is that different cell lines were studied. Our studies have all been performed on one strain of fibroblasts (American Type Culture Collection) originally obtained from a patient with FA. Therefore we cannot yet conclude that the findings are characteristic of all FA cells. Nevertheless, they are interesting since they demonstrate a failure of DNA repair at a site not previously described.

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Introduction of mouse L cell nucleus into heterologous mammalian cells

HARRIS¹ first achieved the introduction of a chick erythrocyte nucleus into other nucleated mammalian cells, without any detectable incorporation of chick erythrocyte cytoplasm, using inactivated Sendai virus. Since then chick erythrocyte nuclei have provided a way of investigating the genetic regulation of nuclear synthetic activities in the presence of a genetically disparate cytoplasm and nucleus^{2,3}. Similar studies with nuclei from other mammalian cells have been presented, however, by the lack of a method for obtaining nuclei suitable for introduction into another type of cells.

When Carter⁴ first reported the enucleation of mammalian cells by the drug cytochalasin B, a metabolic product of the fungus *Helminthosporium dematioides*, he predicted that the separated nuclei and the enucleated cytoplasm might be used in nuclear exchange experiments. Since then the fusion of enucleated cells of one type with nucleated cells of another type has been achieved^{5,6}. During our earlier electron microscopic studies^{7,8} on the enucleation of mouse L cells with cyclochalasin B we observed that the nucleus ejected from the cell carried along with it a very thin rim of cytoplasm within the plasma mem-

brane. As the fusion of cells induced by the inactivated Sendai virus is believed to involve the fusion of two plasma membranes it seemed possible to fuse the nuclei obtained from cytochalasin B-treated cells with other types of cells. Here we report the introduction of mouse L cell nuclei, prepared by treating the cells with cytochalasin B, into nucleated human HeLa cells with the aid of inactivated Sendai virus.

Mouse L cells (clone 929) used for obtaining the nuclei were grown on the surface of plastic Petri dishes as monolayers in Eagle's medium containing 15% calf serum, 100 units ml⁻¹ of penicillin and 100 µg ml⁻¹ of streptomycin (GM). The cultures were prelabelled with tritiated thymidine; before use, 100 µCi of ³H-thymidine was added to the cultures every 6 h for a total of 42 h. This resulted in the labelling of almost every cell. Such prelabelled sheets of L cells were treated with cytochalasin B to bring about enucleation^{4,9}. The method used was a modification of the technique described by Prescott *et al.*⁹.

Briefly, it consisted of inserting plastic Petri dishes carrying adherent cell sheets into stainless steel centrifuge tubes. The tubes contained BM supplement with 1 µg ml⁻¹ of amphotericin B and 5 µg ml⁻¹ cytochalasin B (Serva, Feinbiochemica, Heidelberg) predissolved in dimethylsulphoxide (DMSO) at a concentration of 1 µg ml⁻¹. Centrifugation at room temperature for 45 min at 10,000g resulted in almost complete enucleation. The Petri dishes were inverted on the bottom of the test tubes (cell side facing down) so that the plane of cell layer was at an angle of 37° ± 2° to the line of centrifugal force.

The addition of amphotericin B to the reaction mixture facilitated the cytochalasin B-induced enucleation process (unpublished results). The sediment at the bottom of the centrifuge tubes consisted mainly of L cell nuclei. Figure 1 shows the enucleation phenomenon. Observed under the electron microscope the extruded nuclei reveal the presence of a thin rim of cytoplasm around them containing ribosomes, very rarely one or two mitochondria and a few fragments of endoplasmic reticulum. It was itself enclosed by a plasma membrane (Fig. 2).

The L cell nuclei were introduced into human HeLa cells maintained in GM. These cells (10⁶) were seeded in Leighton tubes containing glass coverslips and incubated at 37° C. After 48 h, the culture medium was removed and the cell sheet was layered with a thin suspension of L cell nuclei and 500 haemagglutinin units of Sendai virus (inactivated with 0.06% β-propiolactone¹⁰). The cultures were

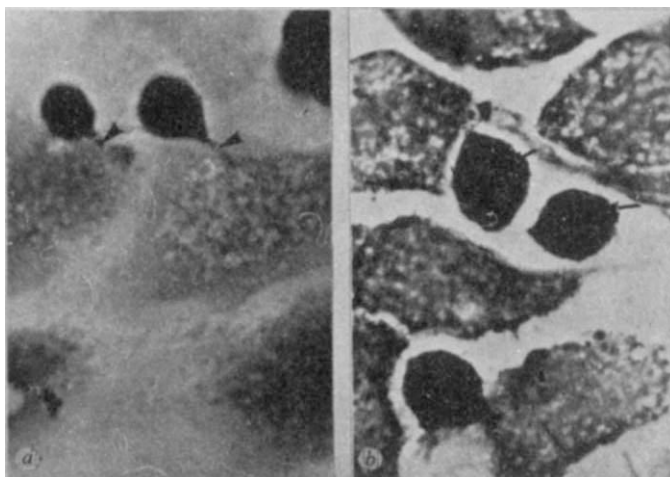


Fig. 1 L cell enucleation by cytochalasin B. a, during extrusion from the cells, nuclei are connected to the cells by thin cytoplasmic processes (arrows); b, Nuclei ejected from the cell cytoplasm show prominent nucleoli (arrows). Giemsa stain ($\times 528$).

kept in the cold for 10 min followed by incubation at 37° C for 1 h. Fresh GM was added after washing off the excess nuclei and inactivated virus. Some of the coverslips were processed immediately for autoradiography but other coverslip cultures were incubated further and examined at regular intervals.

The presence of labelled L cell nuclei near the unlabelled HeLa cell nucleus (Fig. 3b) provided unequivocal evidence of the introduction of the L cell nuclei into HeLa cells. Approximately 10% of the cells were binucleate heterokaryons (Fig. 3a), the remaining being multinucleate heterokaryons, homokaryons or mononucleate HeLa cells. (Heterokaryons and somatic cell hybrids are the terms generally reserved for cells arising following the fusion of two or more genetically different types of cytoplasms and nuclei and as such have been used here.)

The presence of L cell membrane properties on the cell surface of heterokaryons provided evidence for the effective functioning of the introduced L cell nucleus. Mouse species-specific antigens were detected by the mixed haemadsorption technique^{11,12}. L cells carry on their surface a type C virus-induced antigen and this was detected on heterokaryons by indirect membrane immunofluorescence using anti-Moloney virus serum as described by Fenyö *et al.*¹³. HeLa cells showed complete absence of these surface properties.

The multinucleate heterokaryons and homokaryons gradually disappeared from the culture within 1–2 weeks, leaving behind either mononucleate hybrid cells or HeLa cells. The recognition of hybrid cells presented no problem as their cytoplasm was not well spread and the nucleus was frequently larger than the HeLa cell nucleus. These hybrids continue to express some of the L cell surface traits. A detailed account of the studies on the karyotypic analysis of these hybrids and their antigenic properties will be reported elsewhere.

The possible contamination of these hybrids with a small amount of L cell cytoplasm, which remains attached to the L cell nucleus, cannot be excluded. It seems unlikely, however, that such a small amount of cytoplasm in the hybrid will have any controlling effect on the expression of nuclear functions as it would be rapidly diluted out in the hybrid progeny cells.

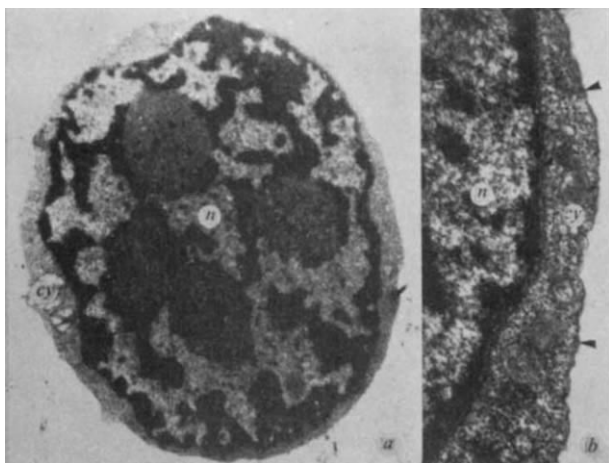


Fig. 2 Electron micrographs of the ejected L cell nuclei. *a*, Ejected L cell nucleus, *n*, surrounded by a thin fragment of the cell cytoplasm, *cy*, containing some of the cytoplasmic organelles ($\times 8,200$); *b*, Enlarged portion of the ejected L cell nucleus showing a distinct plasma membrane (arrows) surrounding the cytoplasmic fragment, *cy*, which in turn encloses the nucleus, *n* ($\times 24,000$).

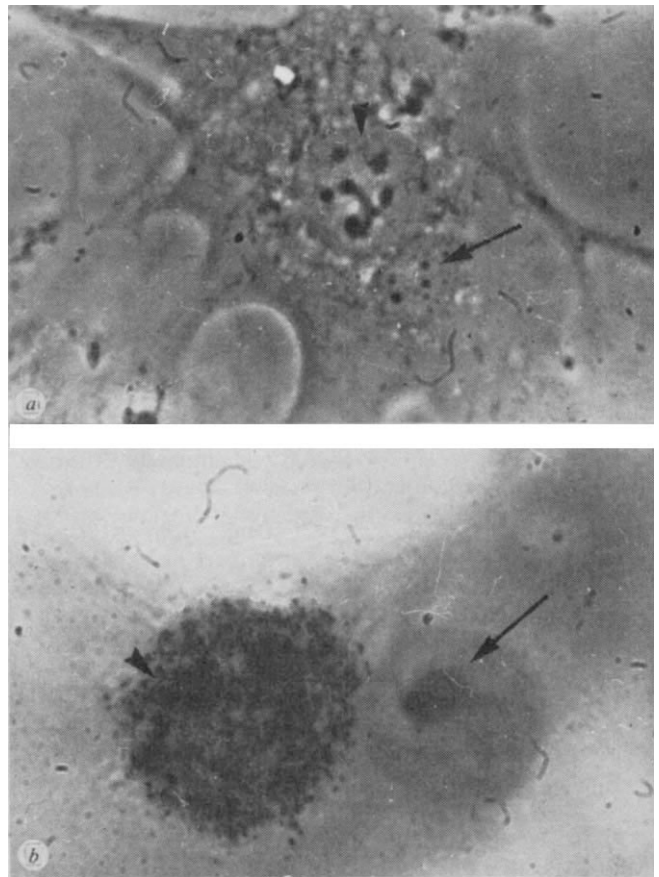


Fig. 3 *a*, Phase contrast photomicrograph of a HeLa cell containing the introduced large L cell nucleus (arrow). Note the relatively small HeLa cell nucleus (long arrow) ($\times 1,125$); *b*, Autoradiograph of a heterokaryon containing unlabelled HeLa cell nucleus (long arrow) and the labelled L cell nucleus (arrow) within HeLa cell cytoplasm. The L cells were grown in ³H-thymidine before the nuclei were removed and introduced into HeLa cells ($\times 1,418$).

Our demonstration of the introduction of L cell nuclei into HeLa cells makes possible the introduction of isolated mammalian cell nuclei into enucleated cells, thereby allowing true nuclear exchange experiments which have been previously impossible with mammalian cells.

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Effect of β_2 microglobulin antibody on effector function of T-cell mediated cytotoxicity

A MEMBRANE fragment of human lymphoid cells obtained by papain digestion has been found to be composed of two subunits which differ in molecular weight^{1,2}. The heavy molecular weight subunit (33,000 daltons) carries the HL-A serologically defined (SD) antigens while the light molecular weight subunit (11,000 daltons) carries antigenic determinants reacting with antiserum directed against β_2 microglobulin (β_2 m)³⁻⁶. Treatment of human lymphocytes with antisera directed against the HL-A SD antigens results in capping of these antigens⁷; addition of β_2 m antibody to lymphocytes will likewise cause capping of the HL-A SD antigens^{8,9}. Furthermore, activation of lymphocytes in mixed leukocyte culture (MLC) can be inhibited by β_2 m antibody^{9,10}. These data suggested to the investigators^{9,10} that the HL-A subunit and/or the β_2 m subunit may be part of the same receptor complex of the T lymphocyte. This concept is supported by the recent studies of β_2 m demonstrating extensive homology between β_2 m and the constant regions of the heavy chain (and the light chain) of immunoglobulin IgG₁^{11,12}. Consequently β_2 m was thought to have a recognition function similar to that of immunoglobulin.

Lymphocytes stimulated by allogeneic cells in MLC specifically destroy target cells autologous to the stimulating cells¹³. Effector cells generated in MLC are thought to be thymus-derived (T) cells¹⁴. We wish to report here that preincubation of MLC-generated effector cells with β_2 m antibody has no effect on their ability to specifically recognise and destroy target cells autologous to the sensitising cells. Preincubation of target cells with anti β_2 m antibody resulted in enhancement of cytotoxicity due to an antibody-dependent cell-mediated cytotoxicity.

The purification of lymphocytes, generation of effector cells in MLC and preparation of ⁵¹Cr-labelled target cells has been previously described^{13,15}. Anti- β_2 m antiserum was prepared in New Zealand rabbits¹⁶. All sera were decapitated by heating at 56°C for 30 min. The anti- β_2 m was rendered specific by absorption with lyophilised human serum, lyophilised urine of normal individuals and erythrocyte membranes. The antiserum gave a single precipitin line on Ouchterlony immunodiffusion plates and immunoelectrophoresis with pure β_2 m and with sera from patients containing large amounts of β_2 m. Addition of purified β_2 m to the antiserum completely eliminated the enhancement of cytotoxicity when the target cells were

preincubated with β_2 m antibody. For preincubation of effector or target cells, a volume of 50 μ l of undiluted β_2 m antiserum or normal rabbit serum was added to 0.1 ml of lymphocytes (2×10^6) and incubated at 4°C for 1 h. The cells were centrifuged (400g) for 10 min, washed three times in tissue culture media 199 (TC-199) and used immediately in the cytotoxicity reaction. The target cells were labelled with ⁵¹Cr before incubation with the β_2 m antiserum.

The cytotoxicity reaction was conducted in microculture plates with 1×10^6 effector cells and 1×10^4 target cells contained in a total volume of 0.2 ml TC-199 supplemented with 20% human frozen pooled plasma. The plate was incubated for 4 h at 37°C in an atmosphere of 5% CO₂ and 95% air. Then the plate was centrifuged, an aliquot removed and ⁵¹Cr release monitored as previously described¹⁵. All reactions were done in triplicate.

As demonstrated in Table 1, lymphocytes of individual A were sensitised in MLC to the histocompatibility antigens of individuals B or C. The degree of stimulation was measured by ³H-thymidine incorporation. The allogeneic combinations AB or AC produced good stimulation while the autologous combination (AA) produced relatively little ³H-thymidine incorporation. After 6 d, ⁵¹Cr labelled lymphoblasts were added to the responder cells (now termed effector cells). Significant ⁵¹Cr release was obtained when the target and stimulator cells were autologous (AB/B). When the stimulator and target were allogeneic (AC/B) no ⁵¹Cr release was demonstrated. In addition, when the responder-stimulator combination was autologous (AA/B) or when the target and responder cells were autologous (AB/A) no ⁵¹Cr release was obtained. Preincubation of the sensitised effector cells with the β_2 m antiserum had no significant effect on the ability of the cells to specifically destroy target cells. Preincubation of the target cells with the same concentration of anti- β_2 m resulted in an enhancement of the cytotoxicity, which was due to an antibody-dependent cell-mediated type of cytotoxicity first described by Moller¹⁷. In this type of cytotoxicity unsensitised lymphocytes perform equally well as effector cells. As demonstrated in Table 1, unsensitised lymphocytes (A) used as effector cells against the target cell (B) produced excellent ⁵¹Cr release. The β_2 m antiserum was used in the experiments reported here at a final dilution of one to three. Complete inhibition of MLC was produced with this same antiserum at a dilution 1 to 50¹⁸.

Antisera directed against either β_2 m or the SD antigens of the HL-A complex inhibit the MLC. In addition, β_2 m and the SD antigens have been shown to be associated on the lymphocyte membrane. These facts, in addition to the homology between β_2 m and the constant domains of IgG, have led several investi-

Table 1 Effect of β_2 microglobulin antibody on effector and target cells

Responder/stimulator	MLC Mean c.p.m. $\pm$ s.d.	Target	Preincubation serum (cells preincubated)	⁵¹ Cr Release*		
				Exp. value mean $\pm$ s.d.	Exp. value minus spontaneous release	⁵¹ Cr Release
AB	31,873 $\pm$ 2,461	B	normal rabbit (target and effector)	3,547 $\pm$ 231	1,980	46.7
AA	795 $\pm$ 382	B		1,591 $\pm$ 84	24	0.6
AB	31,873 $\pm$ 2,461	A		1,533 $\pm$ 125	-34	-0.8
AC	41,954 $\pm$ 3,412	B		1,727 $\pm$ 107	160	3.8
AB	31,873 $\pm$ 2,461	B	anti- β_2 m (effector)	3,641 $\pm$ 54	2,074	48.9
AB	31,873 $\pm$ 2,461	B	anti- β_2 m (target)	2,874 $\pm$ 73	2,156	95.4
AB	31,873 $\pm$ 2,461	B	anti- β_2 m antibodies with β_2 m (target)	1,899 $\pm$ 171	1,181	52.3
A	(unsensitised)	B	anti- β_2 m (target)	2,861 $\pm$ 56	2,143	94.8

* Spontaneous release (c.p.m. $\pm$ s.d.) for B (Preincubated normal rabbit serum): 1,567 $\pm$ 21. Spontaneous release for B (preincubated anti- β_2 m): 718 $\pm$ 34. Frozen thawed c.p.m. for B (preincubated normal rabbit serum): 5,804 $\pm$ 156. Frozen thawed B (preincubated anti- β_2 m): 2,978 $\pm$ 71.

$$^{51}\text{Cr release} = \left[\frac{\text{Expected value} - \text{spontaneous release}}{\text{Frozen thawed} - \text{spontaneous release}} \right] \times 100$$

gators to suggest that the β_2m subunit and the alloantigenic subunit are closely associated with the lymphocyte receptor^{9,10}. Convincing evidence has been put forward demonstrating that the genetic loci responsible for stimulation in MLC are separate from the HL-A loci coding for the SD antigens²¹⁻²⁵. Evidence from several laboratories^{19,20} have suggested that effector cells generated in MLC recognise only SD antigens. Therefore, the receptor sites on the T cells responsible for cell destruction are presumably directed toward the SD antigens. Our data suggest that the receptor sites responsible for the recognition do not seem to be associated with β_2m . In addition, Poulik *et al.* have demonstrated that there is no association between β_2m and surface immunoglobulin on the membrane of human B cells⁸. One cannot rule out the possibility that the receptor which recognises the gene product of the MLC loci is of a different type (that is, associated with β_2m) than that responsible for cell destruction.

The relationship between β_2m and the antigen on the target cell responsible for cell mediated cytotoxicity is unclear because of the interference by the antibody-dependent cell-mediated cytotoxicity. This problem can be eliminated by using F(ab)₂ of the β_2m antibody in the cytotoxicity reaction. Experiments toward this end are in progress.

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New alloantigen genetically linked to the major histocompatibility locus of the mouse

THE mouse *H-2* locus and its genetic homologue, the *HL-A* locus in man, are much more complex than originally envisaged, and are now referred to as the major histocompatibility complex (MHC)¹. Transplant rejection is more closely correlated with mixed lymphocyte reaction (MLR) than with tissue typing²⁻⁶ and in addition to an MLR region in the MHC other regions are of obvious importance in the context of recognition, for example the immune responsiveness regions (*Ir*)^{7,8}. The only gene products of the MHC so far characterised are those recognised serologically by 'tissue typing' antisera, that is, *H-2* antigens and their homologues in other species. These products are not the only substances involved in the fate of allografts; indeed sensitisation against an allograft seems to require MLR differences although serologically detected components have an, as yet undefined, role in the effector arc of rejection⁹⁻¹¹. Though there are suggestions that MHC gene products other than *H-2* (*HL-A*, and so on) may be primarily involved in graft rejection¹², the only *in vivo* studies pointing strongly in that direction were carried out in the rat enhancement model¹³. In these studies rat heart allografts were passively enhanced by alloantisera from which Ag-B (the *H-2* homologue in rats) antibodies had been removed by absorption with appropriate red blood cells (RBC), which suggests a role for antibodies directed against other products of the MHC.

MLR and graft-versus-host reactions may occur in the absence of serological differences at the MHC³⁻⁵ and since genetically engineered situations have led to the detection of new T- and new B-cell associated antigens in the mouse system¹⁴⁻¹⁷, great importance attaches to the definition of other products controlled by the MHC. Here we describe some properties of a gene product found on thymus-derived lymphocytes and determined by a gene near the *K-end* of the MHC.

In a complement-dependent cytotoxicity test, an anti-serum (kxb anti-d) prepared in (B10.Br×C57BL/6)_{F1} mice by immunisation with DBA/2 spleen cells showed, after absorption with B10.D2 or BALB/c (*H-2^d*) RBC, only 25-30% ⁵¹Cr release with B10.D2 lymph node target cells. This target cell is used to provide a congenic system; hence genetic markers not related to the MHC remain undetected. A membrane suspension from BALB/c spleen cells was solubilised by controlled papain digestion, fractionated on DEAE Sephadex-A50, and six peaks eluted stepwise at (1) 0.05 M, (2) 0.1 M, (3) 0.15 M, (4) 0.2 M, (5) 0.25 M and (6) 0.5 M NaCl were collected¹⁸. Fractions 3 and 4 contained the *H-2* activity (*H*-antigens). These six fractions when monitored with the RBC absorbed antiserum, showed a totally different pattern: only fraction 5, eluted with 0.3 M NaCl showed specific inhibition of immune cytotoxicity (160 ID₅₀ units mg⁻¹). An included fraction corresponding

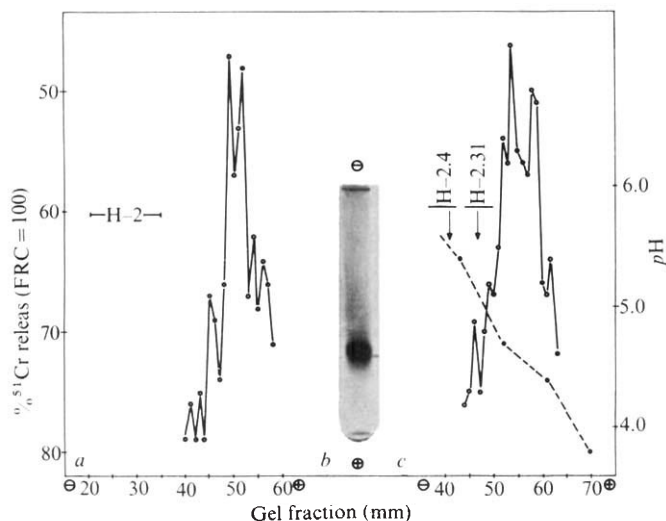


Fig. 1 Polyacrylamide gel electrophoresis. *a*, Serological profile of a highly purified antigen fraction. Gel slices (1 mm) were eluted, dialysed and freeze dried, and the material was tested for alloantigenic activity by inhibition of complement-dependent lysis (⁵¹Cr release). For this purpose an H-2 antiserum was first absorbed with red blood cells from H-2^d mice¹³. Inhibition of immune cytotoxicity (O—O). The position where H-antigens would be found in the same system¹⁹ is indicated by the horizontal bar. *b*, Staining pattern of the new alloantigen; migration is from - to +. The discontinuous system used in the present work has been described in detail previously^{18,19}. *c*, Isotachopheresis of the same fraction, performed as detailed elsewhere^{18,19}. pH measurements were carried out in the gel eluates (1 mm fractions). The position (pH) where H-2 antigens would appear but which were removed by previous DEAE chromatography, is indicated by arrows. A specificity check was performed in parallel (not shown) using a RBC-absorbed B10.D2 anti-B10.A antiserum with B.10A target cells. Inhibition of immune cytotoxicity (O—O); pH (●—●). FRC=full release control, indicating 100% ⁵¹Cr release.

to an apparent molecular weight of 36,000 (1,700 ID₅₀ units mg⁻¹) was isolated by Sephadex G-100 chromatography. On SDS electrophoresis this fraction migrated with an average *M_r* of 34,000. After elution from polyacrylamide gel electrophoresis, a serologically active peak was obtained at a relative migration value of about 0.67, corresponding to a single band in a stained gel run in parallel. For comparison, H antigens migrated in the same system with *R_F* values of about 0.4. A further purification on DEAE Sephadex-A50 provided an active fraction with an electrophoretic and serological pattern as depicted in Fig. 1*a* and *b*. Fig. 1*c* shows the *pI* (pH 4.6) of this substance compared to about pH 5.0 (H-2.31) and 5.4 (H-2.4) for H antigens. Furthermore this material contains 0.57 mol SH per mol protein as determined by DTNB (ref. 20).

Previous results indicated that desorption of papain-solubilised H-antigens reacting with H-2.34 antisera occurred later than the main H-2 fraction on ion exchange chromatography²¹. The reactivity of the RBC-absorbed H-2 antiserum with the new antigen was, however, unimpaired after the antiserum had been further absorbed with DBA/1 (H-2^q) lymph node cells, thus eliminating H-2.34. A genetic linkage to the MHC is clear since the antiserum used was obtained from (B10.Br×C57BL/6)_{F1} mice providing congenicity with the B10.D2 target cells.

When tested for *D-end* activity, two antisera (*K^dD^d* × *K^bD^b*) anti *K^dD^d* (Fig. 2*a*) and (*K^dD^d* × *K^bD^b*) anti *K^dD^d* (Fig. 2*b*) showed no residual titre using *K^dD^d* or *K^dD^d* target cells respectively. This indicates that the residual cytotoxicity observed in Fig. 2*b* for the H-2.31 antiserum and in Fig. 2*c* is a *K-end* reaction and that the antigenic product is controlled by gene(s) located near the *K-end* of the MHC.

It is of particular significance in this context that the *Ir*

region is in the close vicinity of the *K-end* of the MHC⁸. *Ir* gene products have been considered to be candidates for T-cell recognition structures⁷. We would like to propose the term 'R-antigen' for these substances in contrast to

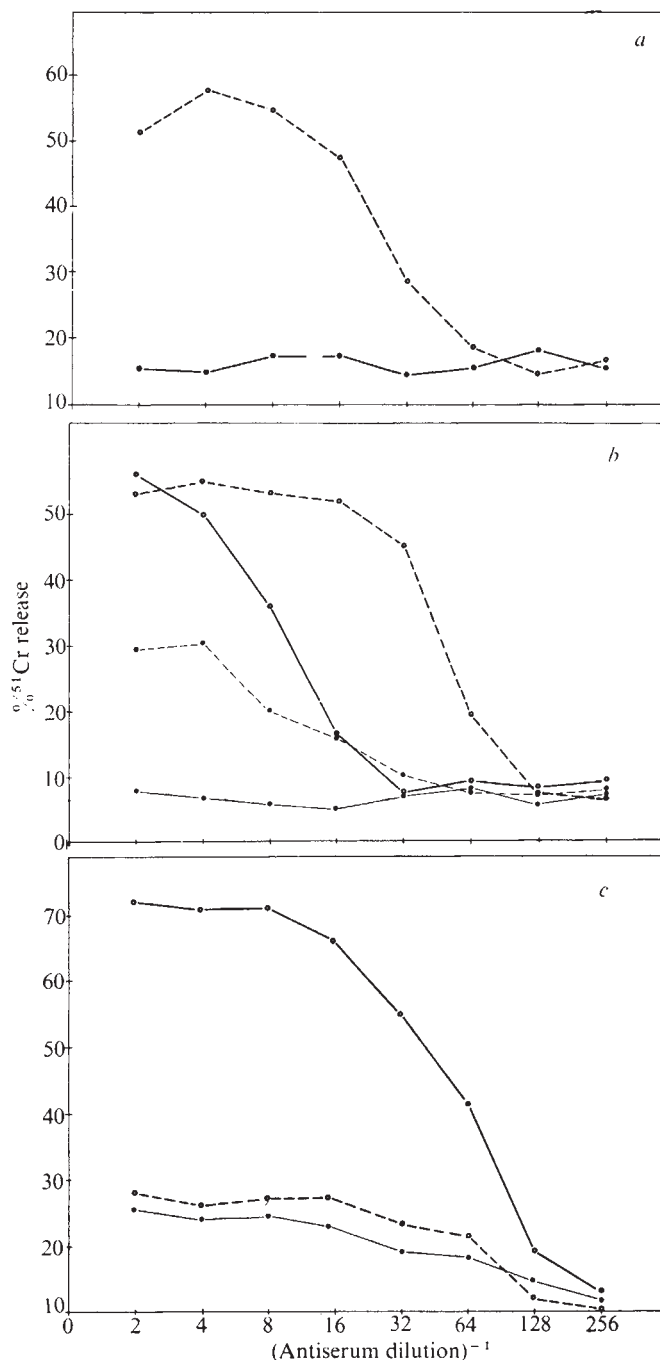


Fig. 2 An antiserum (H-2.4, 10, 13, 31, 34, 40, 41, 42, 43, 44) was prepared by immunisation of *K^dD^d* × *K^bD^b* _{F1} hybrids with (*K^dD^d*) mice. Titres obtained in cytotoxicity test (⁵¹Cr release) using *a*, B10.A (*K^dD^d*) target cells with the H-2 antiserum described above (AS 73/5) after absorption with DBA/1 (*K^dD^d*) lymph node cells (which removes H-2.13, 34), (O - - O) and with AS 73/5 absorbed with B10.D2 (*K^dD^d*) red blood cells (removing the remaining H-2 specificities), (● - - ●); *b*, B10.D2 target cells with H-2.4 (—) and H-2.31 (---) antisera before (O) and after (●) RBC absorption. *c*, Serological pattern of AS 73/5 tested against B10.D2 target cells before (O—O) and after (O - - O) absorption with B10.D2 or BALB/c RBC and after further absorption with DBA/1 lymph node cells (●—●).

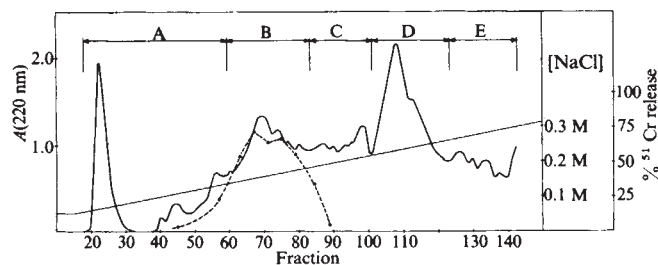


Fig. 3 DE-52 chromatography pattern of a thymus cell membrane preparation solubilised with papain (1:100, 1 h, 37° C) and previously run on a P-300 Biogel column. The DEAE column was developed with a linear NaCl gradient (0.0 M–0.3 M) in a total volume of 750 ml 0.05 M Tris-HCl buffer, pH 8.0. Fractions of 5 ml were collected. The H-2 serological activity (●—●) was measured by inhibition of complement-mediated cytotoxicity (^{51}Cr release) and is shown to be confined to the region of pool B. Pools (A to E) were made as indicated. Note the magnitude of pool D. Using an H-2^d thymus preparation the specific activity (ID_{50} units) obtained with the RBC absorbed antiserum for pool D was 2.5 times (380 U mg^{-1}) the activity recovered from comparable spleen material prepared under identical conditions.

the H antigens mentioned previously. An association of the present antigen with T cells comes from two sources. First, target lymph node cells treated with anti- θ serum, which gave a 50% kill, did not lead to the two-fold titre increase expected if the antiserum were B-cell reactive. Second, when thymocyte membranes were extracted and fractionated by ion exchange chromatography as described previously²², the major component present (pool D) had identical physicochemical and serological properties to the antigen isolated from spleen cells (Fig. 1b and Fig. 3). This despite the fact that under normal conditions thymocytes are not a target for complement-mediated lysis by this antiserum. Thymocytes therefore contain the antigen (tentatively called R1.1) which is exposed, however, only on extrathymic cells. Recently several new specificities not attributable to D- or K-end gene products have been described (see refs 14, 16). Whereas these seem to be B-cell features, the antigenic products we describe here has T-cell affinities. Like other MHC gene products, the biological significance of this new substance remains to be determined.

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Generation of antistreptolysin O activity in contaminated sera

THE ability to lyse erythrocytes of reduced streptolysin O, produced by strains of Lancefield's group A streptococci apparently results from attachment to membrane cholesterol. Cholesterol in aqueous emulsion will inhibit streptolysin activity at concentrations of about $1.0 \mu\text{g ml}^{-1}$ (ref. 1). Sera contaminated with bacteria can occasionally give false positive tests for anti-streptolysin antibody but the underlying mechanism has never been defined. We have excluded the possibility that bacterial contamination leads to release of free cholesterol from either ester-bound or protein-bound cholesterol by showing that (1) cholesterol added to contaminated sera showing positive antistreptolysin activity is bound to the same extent as in normal serum, and (2) that ultrafiltrates of such contaminated sera do not contain free cholesterol. Therefore it seemed likely that bacteria in serum might release proteolytic enzymes which would cleave the streptolysin molecule. But the studies reported here indicate that the system is more complex.

Organisms were subcultured from single colonies on agar plates into 1.0 ml amounts of a horse serum-nutrient broth mixture 2:3 v/v). After incubation at 37° C for various periods, cultures were serially diluted in phosphate-buffered saline (0.15 M; pH 6.5) and antistreptolysin activity measured². Results were expressed as that dilution of culture which gave 50% lysis of added human erythrocytes in the presence of 1.0 IU of streptolysin. Table 1 shows the distribution of positive activity in a number of common genera of microorganisms.

Most strains of *Pseudomonas* were positive (90%). Coagulase positive staphylococci showed an 86% positive rate as distinct from 32% for *Staphylococcus albus*. Strains of Enterobacteriaceae such as *Escherichia coli* and *Proteus* species were uniformly negative as were bacillus species, *Haemophilus*, streptococci, pneumococci and *Candida* species. One positive strain of *Neisseria catarrhalis* was detected and one positive strain of *Clostridium histolyticum*. Staphylococcal strains gave positive results with both aerobic and anaerobic cultures. In all cases positive activity appeared slowly after 36–48 h and was maximal after 4–6 d incubation.

Investigation showed that serum is essential, broth cultures themselves having no antistreptolysin activity. Concentrations of serum less than 2.0% gave poor results,

activity increasing with increasing amounts of serum to an optimum of 40–50%. Beyond this, activity was often reduced, but it seems that this may be related to a reduction in the number of organisms in the cultures. The findings suggest that certain organisms are stimulated by a serum substrate to form an inducible enzyme which converts the substrate to an activated form capable of destroying or of inhibiting the action of streptolysin O. The addition of positive serum–broth filtrates to aliquots of fresh normal serum results in generation of new activity in the added serum. As well as lacking the active principle, broth filtrates do not contain enzyme as shown by their inability to generate activity when added to fresh serum. Preliminary tests have located the active serum fraction in the alpha-1 globulin region but it has not yet been possible to separate the enzyme from serum components. The enzyme, serum substrate and active factor seem to be heat stable at 60° C for at least 2 h. Horse, sheep, human and rabbit sera all gave positive results although sheep serum was slightly less effective.

Table 1 Distribution of antistreptolysin activity in microorganisms. Cultures were tested daily for up to 28 d

Organism	No. tested	No. and percentage showing antistreptolysin activity at 1/100 or greater dilution of culture	Reciprocal of mean dilution showing 50% haemolysis
<i>Staph. pyogenes</i>	69	59 (86)	1,600
<i>Staph. albus</i>	25	8 (32)	1,600
B-haemolytic streptococci	14	0 (0)	—
Pneumococci	17	0 (0)	—
<i>Haemophilus sp.</i>	10	0 (0)	—
<i>Neisseriae sp.</i>	12	1 (8.3)	200
<i>Bacillus sp.</i>	16	0 (0)	—
<i>Proteus sp.</i>	25	0 (0)	—
<i>E. coli</i>	58	0 (0)	—
<i>Pseudomonas sp.</i>	92	83 (90)	1,200
<i>Clostridium sp.</i>	13	1 (7.6)	800
<i>Bacteroides</i>	8	0 (0)	—
<i>Candida sp.</i>	19	0 (0)	—

There is evidence that normal serum contains an inhibitor of the active serum factor produced as above, since active serum–broth filtrates of 4 d cultures added to aliquots of fresh serum gave levels of activity after 5–6 h which were much below that expected from the dilution factor. In each case this was followed, after 2–3 d incubation at 37° C, by a rise in the active serum factor to a level some four to five times greater than in the original 4 d cultures. But addition of young broth cultures of positive organisms to filtrates containing high levels of active factor did not reduce these on further incubation. This suggests that in the original 4 d cultures the growing organisms had inactivated a certain percentage of the serum precursor either directly or by activation of some inhibitor system, but that the activated factor itself was not further affected by growing organisms.

Apart from the intrinsic interest of this serum system which may be more complex than outlined, the distribution of positive and negative strains raises problems which clearly may be related to taxonomy as well as providing a possible epidemiological marker. We are investigating whether this property of certain microorganisms relates in any way to other established biochemical activities, and trying to define more clearly the nature of the reacting components, and to show whether the same mechanism operates for different species. Preliminary studies seem to exclude a role for plasminogen activation to plasmin since plasminogen activated with streptokinase does not seem to affect streptolysin activity. Other possible pathways include activation of a kallikreinogen–kallikrein system by the bacterial enzyme or

activation of some complement intermediate compounds.

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Antibodies recognise specific structures of triple-helical polynucleotides built on poly(A) or poly(dA)

ANTIGENIC determinants of nucleic acids may comprise individual bases or base sequences, as in denatured DNA, or specific conformational features, as in helical forms¹. Experimentally induced antibodies have shown selective reactivity toward double-stranded RNA^{2–6}, RNA–DNA hybrids⁴, or distinct features of poly(G)·poly(C)⁷ or poly(dG)·poly(dC)⁴. Some of these antibodies can be used to identify double-helical RNA in virus-infected cells^{8,9} and to quantitate it in RNA extracted from such cells¹⁰, or to distinguish between double-stranded RNA and RNA–DNA hybrids in mixtures of enzyme reaction products¹¹. Triple-helical poly(A)·2poly(U) can also be distinguished from double-helical forms^{2,3}. Triple-helical regions can form where continuous purine sequences occur¹², and they might be involved as intermediates or as recognition sites in transcription^{13,14}. Thus antibodies that recognise triple-helical nucleic acids are important in the definition of conformation-dependent antigenic determinants, as a model for protein recognition of specific nucleic acid sites, and they may identify such sites in naturally occurring nucleic acids. We describe here such antibodies that can differentiate three-stranded structures built on poly(A) from others built on poly(dA).

Sera were obtained from rabbits immunised with methylated bovine serum albumin complexes¹⁵ of copolymers formed by mixing: poly(A)+2poly(U), or poly(dA)+poly(U) or poly(dA)+poly(dT)+poly(U). The sera were tested with single, double and triple-stranded polynucleotides. Sera that reacted with free poly(A) or denatured DNA were passed through columns of DNA bound to Sepharose¹⁶. These absorbed sera, and all others used did not react with native or denatured DNA or with poly(A), poly(U), poly(I), poly(C), poly(dA) or with ribosomal or transfer RNA or double-stranded reovirus RNA, poly(I)·poly(C), poly(dA)·poly(dT) or poly(A)·poly(dT).

Since poly(A) and poly(U) may combine to form either a double-stranded or triple-stranded helix^{17,18}, the specificity of the anti-poly(A)·2poly(U) sera for the triple-helical form was tested when homopolymers were mixed in varying proportions. For reaction with these sera, the maximal amount of effective antigen was present when the ratio in the mixture was 2poly(U):1poly(A) (Fig. 1). When equal amounts of homopolymers were mixed, with the double-stranded form predominant, only 5–10% as much reactive antigen was present. The increase in the amount of effective antigen when the poly(U) was increased from 50% to 67% reflected the transition from a double-stranded to a triple-stranded structure^{17,18}, and was the opposite pattern to that

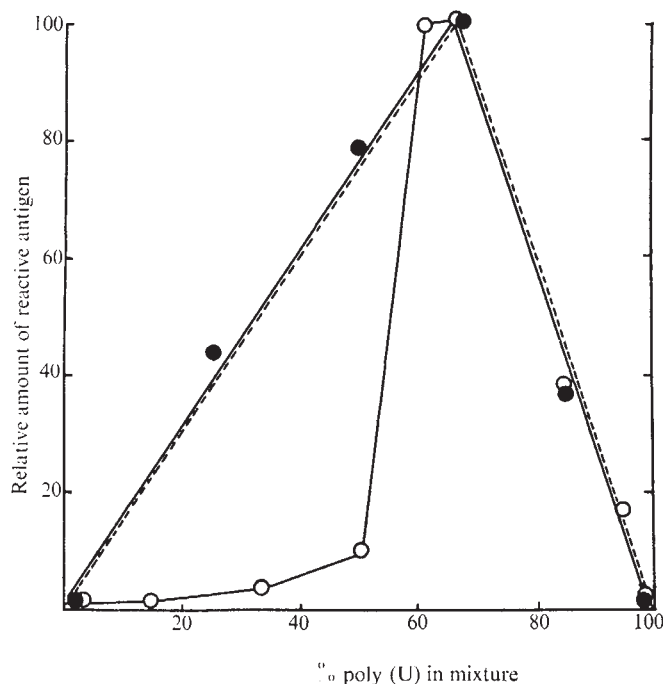


Fig. 1 Specificity of antisera for triple-stranded polymers, measured by formation of reactive antigens in varying mixtures of poly(U) with poly(A) for anti-poly(A)·2poly(U) antiserum (○) and of poly(U) with poly(dA) for anti-poly(dA)·2poly(U) antiserum (●). Homopolymers (Miles) were mixed at concentrations of 1×10^{-5} M nucleotide in 0.14 M NaCl, 0.01 M Tris, pH 7.4, with 5 mM MgSO_4 and 1.5 mM CaCl_2 . Each mixture was diluted and assayed in quantitative micro-complement fixation as described before²³, but in the above Tris-saline buffer, in a total volume of 1.4 ml per reaction mixture. A complement fixation curve was obtained for each polynucleotide mixture. Relative amounts of reactive antigen were calculated from the total polynucleotide required to reach a given point on the complement fixation curve, such as 50% complement fixation in the antibody excess region. Anti-poly(A)·2poly(U) serum was used at a 1/2,000 dilution and anti-poly(dA)·2poly(U) serum at a 1/1,000 dilution. The broken line represents the maximal theoretical value expected if only triple-helical copolymer is formed.

observed when antibodies to a double-stranded form were used with the same mixtures¹. Further, the anti-poly(A)·2poly(U) serum did not react, even at a 1/100 serum dilution, with double-helical poly(I)·poly(C) or reovirus RNA, while it reacted at a 1/5,000 dilution with the homologous poly(A)·2poly(U). Its reaction with homologous antigen was not inhibited by a hundred-fold excess of native DNA, poly(dA)·poly(dT), poly(A)·poly(dT) or poly(I)·poly(C). Thus this serum was specific for the triple-helical polyribonucleotide.

Antisera were obtained from rabbits immunised with a copolymer made by mixing equal amounts of poly(dA) and poly(U). In this case the original serum reacted, at a 1/1,500 dilution with homologous antigen and at a 1/100 dilution with poly(A) and denatured DNA. After it was passed through a DNA-Sepharose column, it no longer reacted with poly(A) or denatured DNA, but was still fully reactive with homologous antigen.

Mixtures of poly(dA) and poly(U) form only a triple-stranded helix¹⁹, so that even if they are mixed in equal amounts, some poly(dA)·2poly(U) and free poly(dA) are formed. This may have been the case with the immunogen, and could explain the separate antibody population that reacted with poly(A) and denatured DNA and was removed by absorption.

When poly(dA) and poly(U) were mixed in varying proportions, the maximum amount of reactive antigen was again

formed when the ratio was 2poly(U):1poly(dA). However, the pattern differed from that seen with the anti-poly(A)·2poly(U) serum, since in this case there was a continuous increase of reactive antigen as poly(U) was added to poly(dA) (Fig. 1) rather than a sudden increase when the poly(U) was increased above 50%. This supports the conclusion that only the triple-stranded structure is formed.

This serum did not react with the double-helical structures listed earlier, and its reaction with homologous antigen was not inhibited by native DNA, poly(dA)·poly(dT), poly(A)·poly(dT) or poly(I)·poly(C).

Antisera to poly(dA)·poly(dT)·poly(U) were also specific for the triple-stranded form reported to occur with this mixture of homopolymers^{19,20}. The sera did not react with the homopolymer components alone or with poly(dA)·poly(dT) or any of the other single or double-stranded polymers listed earlier.

The specificities of these sera were examined further in tests of reciprocal cross-reactivities among the triple-helical polynucleotides. Even at a 1/100 serum dilution, antiserum specific for the exclusively polyribonucleotide copolymer poly(A)·2poly(U) was not cross reactive with poly(dA)·2poly(U) or with poly(dA)·poly(dT)·poly(U); it was effective with homologous antigen at a 1/1,500 serum dilution.

Antisera to the two forms containing poly(dA) were mutually cross-reactive, giving virtually identical complement fixation curves with either antigen (Fig. 2), but neither cross-reacted with the poly(A)-containing triple-helix. The apparent cross-reactivity of poly(dA)·2poly(U) and poly(dA)·poly(dT)·poly(U) was further examined to determine whether when poly(U) was added to poly(dA)·poly(dT), it simply displaced the poly(dT) to form poly(A)·2poly(U). Specific precipitates were formed with anti-poly(dA)·2poly(U) serum and with either of the poly(dA)·2poly(U) or poly(dA)·poly(dT)·poly(U) antigens. The latter precipitate contained all three nucleotides (Fig. 3). Thus the antiserum did precipitate a polymer containing all three components and the two triple-helices must have similar conformations.

The cross-reaction described above indicated that the presence of the poly(dA) controlled the overall configuration of the two similar polymers, while the presence of poly(A) determined the different conformation of the poly(A)·2poly(U). This role of polypurine was tested further by

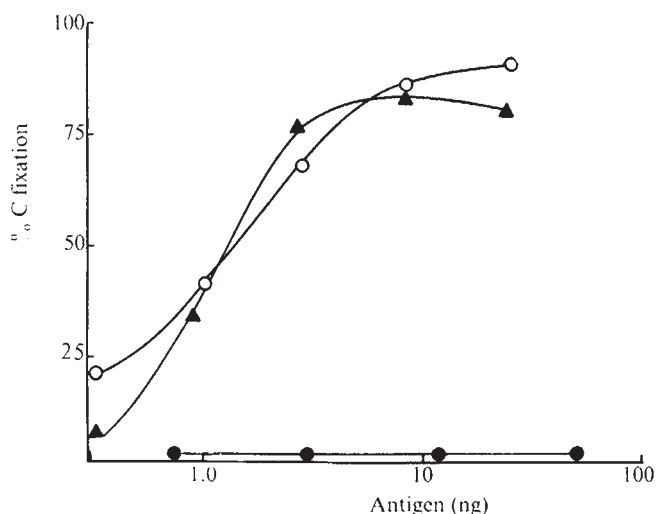


Fig. 2 Cross-reactions of triple-helical polynucleotides with anti-poly(dA)·2poly(U) antiserum. Quantitative complement fixation reactions of poly(dA)·2poly(U) (○) and poly(dA)·poly(dT)·poly(U) (▲) with a 1/1,000 dilution of serum and the lack of reactivity of poly(A)·2poly(U) (●) with a 1/100 dilution.

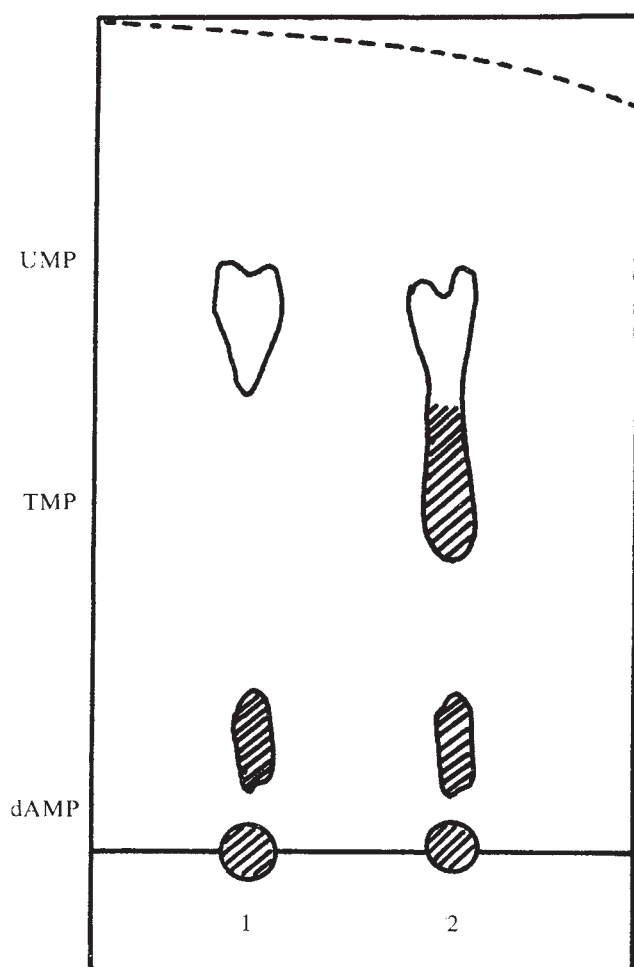


Fig. 3 Analysis of precipitates formed with anti-poly (dA)·2poly(U) antiserum and poly(dA)·2poly(U) or poly(dA)·poly(dT)·poly(U). Poly(dA)·2poly(U) was formed by mixing 4.5×10^{-8} mol of poly(dA) nucleotide with 9×10^{-8} mol of poly(U) nucleotide in 0.3 ml containing 0.3 M NaCl, 0.01 M phosphate, pH 7.4. The mixture was heated at 100°C and slowly cooled to room temperature and then to 4°C . Poly(dA)·poly(dT) was prepared in the same way with a mixture of 4×10^{-8} mol of nucleotide for each homopolymer. An identical amount of poly(U) was then added in 0.15 ml of saline-phosphate at 37°C , and the mixture was cooled to 4°C . Anti-poly(dA)·2poly(U) antiserum (1 ml) was added to each of the two polymer mixtures, and these were incubated for 20 h at 4°C . Both precipitates were collected by centrifugation in the cold, drained, washed with 0.5 ml of saline-phosphate, centrifuged again and resuspended in 0.2 ml of water. The suspension was incubated with $5\mu\text{g}$ of pancreatic RNase for 3 h at 42°C and most of the precipitate dissolved. Pancreatic DNase ($10\mu\text{g}$) and venom phosphodiesterase ($50\mu\text{g}$) were added and the mixtures were incubated for a further 2.5 h at 37°C . The mixture was lyophilised and redissolved in 0.025 ml of water. Of this, 0.01 ml was spotted on an Eastman thin-layer chromatogram with a fluorescent indicator background, and chromatographed in a propanol-ammonium sulphate-phosphate solvent system²⁴. Nucleotides were localised under ultraviolet light, and deoxyribose was localised by a cysteine-sulphuric acid spray²⁵, in comparison with known nucleotide standards that were run in parallel with the hydrolysate. Sample 1 was the hydrolysed precipitate formed with poly(dA)·2poly(U) and sample 2 that formed with poly(dA)·poly(dT)·poly(U). The hatched areas indicate the presence of both ultraviolet-detectable and acid cysteine-reactive material. Most of the latter was in the position of dAMP or, in sample 2, of dTMP as well. Some remained at the origin; in control experiments, protein stained in this way and stayed at the origin.

adding poly(U) to the hybrid poly(A)·poly(dT), which did not react alone with antibody to triple-stranded poly(A)·2poly(U). As poly(U) was added, new complexes formed and reacted strongly with the serum (Fig. 4a). At the same time, the formation of the new complexes was reflected in a reduction in double-stranded antigen available to react with specific anti-hybrid antibody (Fig. 4b). If the third strand were situated in the major groove of the original double helix, as with poly(A)·2poly(U)²¹, it may sterically block some of the double-stranded determinants while forming the triple helix. Alternatively, the addition of the third strand may have altered the conformation of the original double helix. In either case, the effect was not total, as 20–30% of the poly(A)·poly(dT) determinants remained accessible to the anti-hybrid antibodies, as judged from the degree of lateral shift in the complement fixation curve (Fig. 4).

The new complexes of poly(A)·poly(dT)·poly(U) did not react with antisera to either of the poly(dA)-containing helices. Thus the two triple-helical polynucleotides formed with poly(A) were immunologically similar to each other but distinct from the triple-helical polymers formed with poly(dA), while the latter two were very similar to each other.

The structure of the antigenic determinants cannot be defined precisely, but the backbones of two or three strands probably contribute to one determinant, and they must be in the proper orientation with respect to each other, because antibodies reactive with poly(dA)·poly(dT)·poly(U) did not react with poly(dA)·poly(dT) or poly(A)·poly(dT)·poly(U). Similarly, antibodies to double-helical poly(A)·poly(dT) may fail to react with other helices containing one of these chains, such as poly(A)·poly(U) or poly(dA)·poly(dT). Again it seems that portions of both helices, in proper orientation, form one determinant. With several antigen systems, the maximal size of the corresponding antibody combining site is about $7 \times 12 \times 35\text{\AA}$ (ref. 22). This could encompass a full helical turn or the full width of the

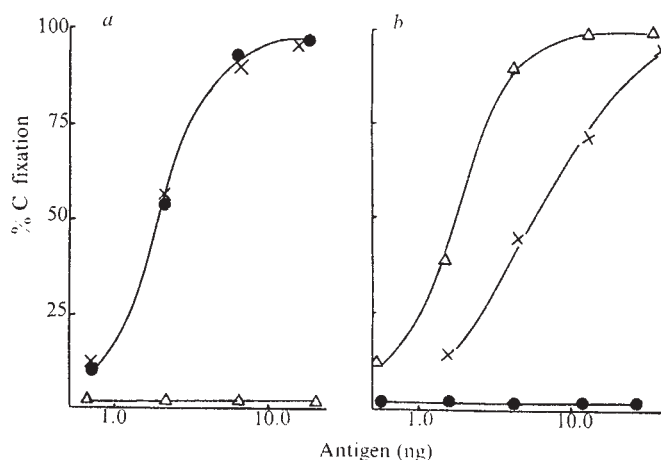


Fig. 4 Complement fixation cross reactivity of poly(A)·2poly(U) and poly(A)·poly(dT)·poly(U) with: (a) anti-poly(A)·2poly(U) antiserum and (b) anti-poly(A)·poly(dT) antiserum. The poly(A)·2poly(U) (●) was prepared by mixing 1 volume of poly(A) with 2 volumes of poly(U), with both polymers at a concentration of 0.4×10^{-7} mol of nucleotide per ml in 0.15 M NaCl, 0.01 M phosphate, pH 7.4, at 37°C . Triple-helical complexes (X) were formed by the addition of 1.2×10^{-8} mol of poly(U) nucleotide to 1.6×10^{-8} mol of poly(A)·poly(dT) (Δ) nucleotide, in 0.025 ml, in 0.4 M NaCl; the mixture was incubated with complement fixation buffer. The anti-poly(A)·2poly(U) antiserum was used at 1/1,000 dilution for reaction with triple-stranded forms and at 1/100 was not reactive with poly(A)·poly(dT). The anti-poly(A)·poly(dT) antiserum was used at 1/1,000 dilution.

helix, including portions of three adjacent strands, in a shallow cavity or groove on the antibody molecule.

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Effect of host decompensation on homeostasis of antibody production in fowl

THE primary response to human serum albumin (HSA) in the 6 to 10-week-old fowl is characterised by an early and accelerating rise in antibody level to a peak at about 9 d followed by an abrupt decline¹. Comparison of the kinetics of this decline from the peak gave a close correspondence between it and the curve of decline of passively transfused labelled homologous 7S immunoglobulin. It seems clear that antibody production has been switched off before the peak and little ensues thereafter. The primary haemagglutinin response to sheep erythrocytes has a similar shape but the peak is earlier (Fig. 1)². The *in vivo* suppressive effect is attributed to the negative feedback inhibition of the 7S

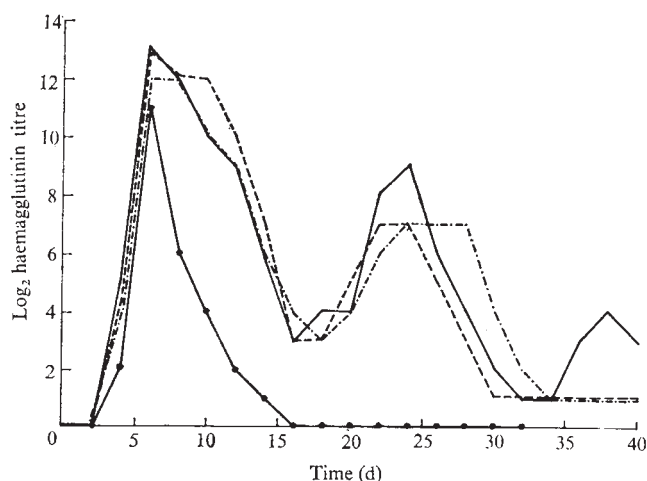


Fig. 1 Anti-sheep RBC response in chickens treated with CoF. Plot of haemagglutinin levels ($\log_2$ titre) following an intravenous injection of 10^{10} sheep erythrocytes. ●—● The average haemagglutinin titres for a group of 10 normal birds. The response reaches maximum at 6 d and thereafter rapidly declines, remaining at or near zero after 16 d. — — — — — Haemagglutinin titres of individual birds treated with cobra venom factor (CoF). The first haemagglutinin response of all birds is slightly increased in comparison with controls at the peak and delayed in the decline phase; all birds also show a further peak (maximum at day 24-25). In the case of one bird a further (third) peak developed (maximum at day 38).

antibody product and is thought to operate by the process of segregation of B cells into germinal centres³.

We have investigated the O-agglutinin response to an intravenous injection of 10^9 heated *Salmonella adelaide* O organisms (Fig. 2). The agglutinin response kinetically resembles that to HSA or sheep erythrocytes, but the initial peak and decline is followed by at least four further peaks of declining magnitude. The class of immunoglobulin involved was investigated by treatment of serum samples with 0.3 M 2-mercaptoethanol for 1 h; the results showed that all agglutinin titres were reduced near to zero. Thus, most antibody was 19S immunoglobulin. The occurrence of 7S chicken agglutinins for *S. adelaide* O antigen was further investigated by use of radiolabelled antibodies prepared against chicken 7S and 19S immunoglobulin. Such antisera, shown to be specific for H chains by standard immunoelectrophoresis, were added to washed agglutinates of *S. adelaide* (heated O organisms) prepared in antigen excess, with 25 μ l of neat chicken serum. After 30 min interaction the uncombined radiolabelled immunoglobulin was removed by washing in physiological (0.15 M) NaCl, and the radioactivity remaining attached to the agglutinates was counted. The counts expressing amounts of 19S antibody are shown in Fig. 3 (range of four peak levels 40,000-82,000 c.p.m.). While estimates of antibody of 19S Ig class in serum samples reproduced the pattern of the previously described agglutination tests, counts of absorbed anti-7S Ig were low (range 100-2,000 c.p.m.) confirming the virtual absence of 7S antibody.

The difference between the curve of agglutinins of the serum response to *S. adelaide* O antigen and the antibody responses to HSA and sheep erythrocytes, is attributed to deficient 7S antibody production in the bird in response to an immunogenic stimulus to the lipopolysaccharide O antigen (see also data from the mouse³). It is hypothesised that normal negative feedback of the primary response requires switch over from 19S to 7S immunoglobulin, without which a single injection of antigen causes a series of cyclical fluctuations in 19S antibody.

Antibody responses to bacterial polysaccharides are unique in several ways. Thus, they depend exclusively on the B-cell

system. Lipopolysaccharide O antigens interact with complement and activate the bypass mechanism of the complement cascade⁵.

This leads to the postulate that the switch over from 19S to 7S biosynthesis of antibody may be a complement-dependent process which is selectively inactivated by lipopolysaccharide antigens. Experiments were undertaken in birds which were immunised with a normal immunogenic stimulus (sheep erythrocytes) during a period of treatment with cobra venom factor sufficient to reduce *in vivo* levels of complement. Cobra venom factor (CoF) was prepared by a modification of previous methods^{6,7}.

One unit of anticomplementary activity was taken as the amount required to reduce the haemolytic capacity of chicken serum by 50%. The preparation of CoF reduced complement levels in the above test 160 fold. It was injected (150 U kg^{-1}) every other day according to the schedule recommended in ref. 8.

The response to a single injection of 10^{10} sheep erythrocytes now took the form shown in Fig. 1. The peak of the first rise of serum agglutinins is elevated (from average titre of 2^{10} to 2^{12-13}). The decline phase is somewhat shifted to the right and in all three cases there is a second rise and fall of serum agglutinins which reaches a peak on day 24 at 2^{7-9} titre. In the case of one bird this cycle is repeated once more, the titre reaching 2^4 on day 38. Another consequence was that virtually all antibody was in the form of 19S immunoglobulin without 7S immunoglobulin. The treatment of sera with 0.3 M 2-mercaptoethanol always reduced to near zero the agglutination titre of serum samples of the three treated with CoF shown in Fig. 1. In control birds, agglutinin titres in the period 5–15 d were also considerably reduced by 0.3 M 2-mercaptoethanol. All the reduced sera were treated to determine the presence or absence of incomplete haemagglutinins by addition of rabbit anti-chicken 7S immunoglobulin. In control birds this caused an increase of the peak titre from 2^2 to 2^{10} , the level declining thereafter. The sera of CoF-treated birds failed to show any appreciable increase following addition of rabbit anti-chicken 7S immunoglobulin.

Treatment with CoF, therefore, acts to impede the normal homeostatic mechanisms which terminate the antibody response to sheep erythrocytes. As this effect was accompanied by a defect of the normal switch from 19S to the 7S immunoglobulin form of antibody, an analogy can be drawn with the situation obtaining in the bird's response to *S. adelaide* O organisms. Here, too, the switch from 19S to 7S antibody fails to occur, and presumably this relates to some distinctive property of this type of antigen. Similarly, it was found that the antibody response to the H antigen of *S. adelaide* failed to terminate after the first peak of antibody (at 5–7 d) but underwent repeated cycles of antibody production of slowly diminishing magnitude.

Pepys^{9,10} has found that treatment of the mouse with CoF results in decrease and/or delay in the antibody responses to two thymus-dependent antigens (sheep erythrocytes and alum-precipitated ovalbumin) but no change in the responses

to two other thymus-independent antigens (pneumococcal polysaccharide type III and polyvinyl pyrrolidone). According to Pepys, the possibility that the CoF effect is due to antigenic competition is unlikely as the use of CoF in previous recipients of CoF cancelled the effect—circumstances in which antigenic competition would have been augmented. It was proposed that complement plays a role in T and B cell cooperation.

Our results can be interpreted as a conversion by CoF of the normal response of a thymus-dependent antigen (sheep erythrocytes) into one with the characteristics typical of a thymus-independent antigen. Both the O (lipopolysaccharide) and H (flagellar) antigens of *S. adelaide* are typical thymus-independent antigens^{11–13} in other animals.

A possible hypothesis to account for our present findings is that lipopolysaccharide and other thymus-independent antigens fail to switch 19S to 7S antibody production and fail to secure lasting homeostasis of the response since they activate the alternative pathway and lead to $\text{C3} \rightarrow \text{C3b} \rightarrow \text{C3b}$ (inactive) conversion⁵.

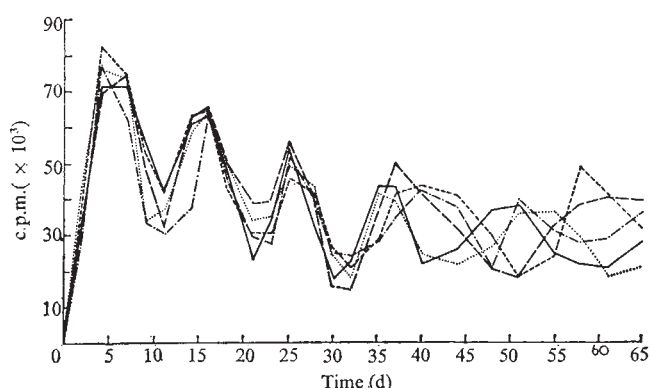


Fig. 3. Plot of antibody levels estimated by radio-immunoassay for individual birds injected with 10^8 *S. adelaide* O. organisms. The response consists of a regular series of separated peaks, each followed by a phase of decline. At least five peaks are recognisable for each bird. Each plot represents the response of one bird.

Finally, it becomes necessary to investigate whether a T-independent response owes its characteristics (recycling of antibody production and lack of 7S response) to a positive anticomplementary action which causes an ineffective switch from 19S to 7S antibody production and defective homeostasis secondarily to a lack of avid 7S antibody. The consequences of this hypothesis can readily be tested and we hope to report the results of such investigations at a later date.

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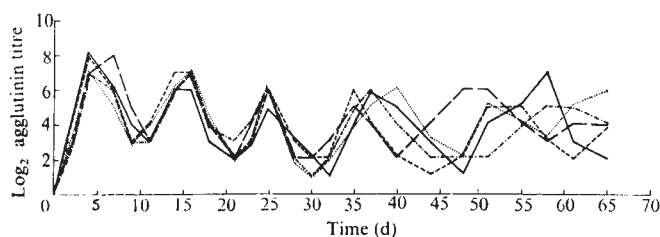


Fig. 2. Plot of agglutinin levels ($\log_2$ titre) for individual adult birds injected with 10^8 *S. adelaide* O. The initial response has a peak at 4–7 d followed by a rapid decline. At least four further cycles of antibody response are shown by all birds. Each plot represents the response of one bird.

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Vaccinia virus cytotoxin(s)

THE mechanisms whereby cytopathic viruses damage or kill susceptible cells are not fully understood. Some experiments with pox viruses involving partially inactivated virus or various metabolic inhibitors suggest a correlation between the production of virus proteins and the appearance of cytopathic effects without necessarily establishing a casual relationship between the two events^{1,2}. Other work suggests that inhibition of host protein synthesis, and by extension induction of cytopathic effects by vaccinia virus, is independent of virus-induced protein synthesis but is some function of the virus particle itself³. Here, we report direct experiments in which vaccinia-specific materials were isolated from infected HeLa cells, rendered free from infective virus and shown to be toxic to susceptible uninfected HeLa cells under appropriate test conditions. To our knowledge this is the first documented example of an observable cell-killing effect of vaccinia specific protein(s). It differs from that of the penton capsomere (toxin) of adenovirus which affects cell membranes from the outside causing them to detach from glass⁴, in that we believe it acts intracellularly.

HeLa cells were infected with vaccinia virus and collected 48 h after infection; soluble virus-free preparations (HVSA) containing both viral and hostspecific precipitinogens were prepared⁵. Preliminary work confirmed previous observations^{1,6} that HVSA had no effect on its own when added to cultures of HeLa cells either in suspension or in monolayers. This meant that either no virus-induced toxic substances existed, or, that they did and were not being taken up by the cells. To test the latter possibility a suitable biological system was sought in which cells could be induced to take up macromolecules in a biologically active state. Various membrane modifiers or uptake inducers⁷⁻¹¹, were tried but none proved more effective or easy to handle than hypertonic salt solutions.

For the test system, HeLa cells were assigned to four groups: group 1 was treated with calf serum medium, group 2 with calf serum medium and MgSO₄ (at the maximum concentration which resulted in $\geq 95\%$ cell survival; see Fig. 1a), group 3 with uninfected cell extract and MgSO₄, and group 4 with HVSA and MgSO₄. Several hours after treatment, some of the HVSA-treated cells were dead as judged by vital staining; after overnight incubation when dead cells had detached and disintegrated, viable cells were stained and counted. The results of 23 experiments (Fig. 1b) show that there is in HVSA, something which is lethal to approximately 30% of HeLa cells present in the test population.

To determine the specificity of this cytotoxic mechanism HVSA was fractionated, using -S-S- linked immunosorbents^{12,13} derived from sera⁵ raised to uninfected HeLa cells or sera from rabbits infected with rabbit-grown vaccinia virus, to yield HeLa- and vaccinia-specific fractions, each overtly uncontaminated by the other in immunodiffusion tests. HVSA was also fractionated on DEAE-cellulose to yield a toxic fraction (Fig. 2) which contained several demonstrable vaccinia antigens in immunodiffusion tests. Only vaccinia-specific fractions were toxic; uninfected cell

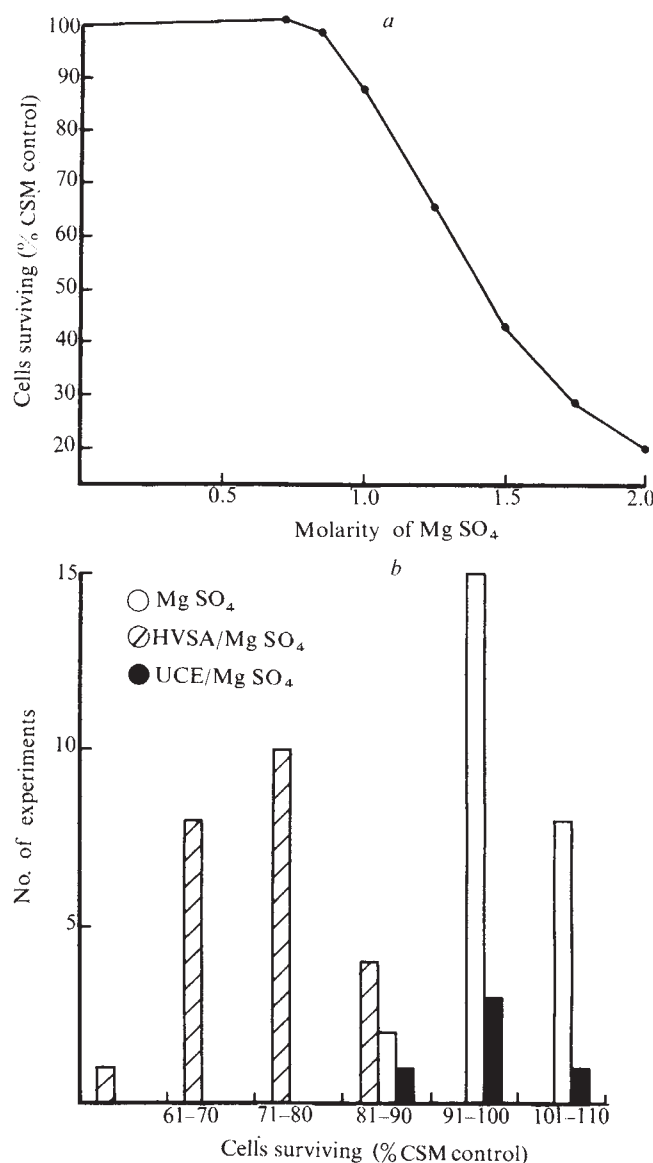


Fig. 1 Survival of HeLa cells treated with MgSO₄, infected cell extract (HVSA)/MgSO₄, and uninfected cell extract (UCE)/MgSO₄, expressed as a percentage of a calf serum medium (CSM)-treated control. Cells from 4 to 6-d-old monolayers were collected with EDTA, counted, diluted in CSM and continuously stirred. Samples (0.2 ml) were pipetted into the wells of a microtitre plate (Flow, M29-ART) so as to give 300-400 cells per well, and the plates centrifuged at 150g for 10 min. Plates were incubated overnight (37° C, 5% CO₂ in air). CSM was removed by inverting the plate, subjecting it to a sharp wrist flick, and removing the residual fluid by placing the plate on sterile filter paper. The cells were treated as follows: *a*, Varying concentrations of MgSO₄ in CSM were added and the cells subjected to the manipulative/counting procedures described in *b*. The object was to determine the highest concentration of MgSO₄ which $\geq 95\%$ of the cells would tolerate, that is, cells would go on to divide upon being returned to CSM. In this case it was 0.85 M MgSO₄, and this was used in the test as described below. Every line (or subline) of HeLa cells used was examined for MgSO₄ sensitivity. This only varied significantly when HeLa cells of a different source or cells obtained by certain selection pressures are used. When one line of normal cells is used the variation from day to day was minimal. *b*, For the test, the following mixtures were added: 0.85 M MgSO₄ in CSM, HVSA (or UCE) mixed with an equal volume of 1.7 M MgSO₄ in CSM, or CSM alone as a control. The cells were treated for 15 min at 37° C, washed twice in 0.2 ml volumes of CSM by the method described above and incubated in 0.2 ml of CSM overnight. Twenty-four hours after treatment the cells were

washed in phosphate buffer (PBS, pH 7.0, I , 0.26), stained for 15 min in freshly filtered 1% crystal violet in PBS Dulbecco A, washed in PBS, stained for 5 min in Lugol's iodine, and washed in PBS again. The wells were filled with PBS and covered with a piece of glass to avoid a refracting meniscus and the cells were observed using the $\times 4$ objective of a Prior Inverted microscope. Cells in the four corners of each well were counted representing about 80% of the area of the well; counts of about 500 cells per well were obtained with a standard deviation of 36 for CSM-treated controls. Routinely 8 or 12 wells were used for the CSM or $MgSO_4$ controls and four wells (to conserve materials) for the HVSA and UCE samples. The majority of tests resulted in approximately 70% of the cells surviving the combined $MgSO_4$ +HVSA treatment, where the control for $MgSO_4$ -treated cells was $99 \pm 6\%$ of the CSM control. Histogram shows data from 23 such experiments involving HSVA with, in brackets of 10%, the frequency with which a given survival of cells was observed. The data for $MgSO_4$ only treatment is derived from the same experiments and is given to show the mildness of this treatment. A similar spread of results for CSM treated cells is obtained when these are measured as a percentage of the $MgSO_4$ -treated control.

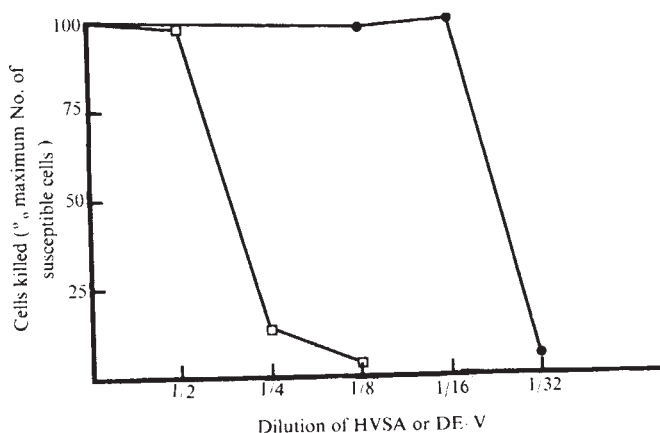


Fig. 2 Titration of toxicity of crude HVSA and partially purified vaccinia-specific antigens. HVSA (1.0 ml) was adsorbed to DEAE-cellulose (phosphate buffer pH 6.8, I , 0.0175) and eluted with stepwise increments of NaCl. The only toxic fraction (DE-V) was that eluted by 1.0 M NaCl and this was concentrated to 1.0 ml and titrated. Titres were expressed as the highest dilution causing death of the maximum number of susceptible cells in the system. Representative results are shown above. Initial concentrations of protein for HVSA □, and DE-V ●, were 20 and 2 mg ml⁻¹ respectively, representing a 10-fold purification. The total toxic activity expressed by DE-V was thus eight times greater than that of crude HSVA per unit volume and 80 times greater per mg protein.

extracts and HeLa materials from infected cells were not toxic in this test. The possibility that some virus-specific component allowed the cells to take up a toxic dose of $MgSO_4$ during the (technically convenient) simultaneous exposure of cells to $MgSO_4$ and HVSA (or vaccinia-specific fractions) was examined by pretreating cells with $MgSO_4$, removing $MgSO_4$, and adding HVSA; the latter still exhibited toxicity, thereby eliminating this possibility. The only biochemical activity associated with HVSA that we have demonstrated so far is the ability to release lysosomal enzymes¹⁴ from rabbit liver lysosome preparations. The level of pre-released β -glucuronidase (the marker enzyme chosen for assay) in uninfected cell extract was twice that in HVSA (due presumably to the loss of enzymes into the medium from the necrotic cells from which HVSA is prepared), but only HVSA caused the release of more enzyme (20–70% of maximum releasable by Triton-X100) from intact lysosomes.

Attempts to titrate toxicity showed that the activities of neat HVSA and 1/2 dilutions of HVSA were approximately the same as judged by the number of cells killed (approximately 30% in each case) and at higher dilutions the activity dropped rapidly to zero. This seemingly low activity may reflect the presence of inhibitors in HSVA. For example, comparative titration of HVSA (20 mg protein ml⁻¹) and the DEAE-cellulose toxic fraction (2 mg protein ml⁻¹) showed the latter to be eight times more active per unit volume or 80-fold more active per mg protein (Fig. 2). All these estimates of toxicity are conservative because activity dropped rapidly to zero. This seemingly low activity apparently stores indefinitely at -20° or -70° C, was slowly inactivated at 37° C, but in the presence of 0.85 M $MgSO_4$, all toxic activity was abolished after 15 min incubation at 37° C.

Preliminary experiments have been carried out in which HVSA was treated with a solid-phase protease (trypsin linked to Enzacryl AA, Koch-Light). This resulted in a loss of toxicity which, together with the fact that immunosorbents specific for vaccinia antigens removed the toxicity from HVSA, suggests that the active factor(s) is a protein and not infectious nuclei acid¹⁵ or double-stranded RNA¹⁶.

Various approaches have been tried, with crude HVSA, to discover why only 30% of the cells were killed. Presumably there is a subpopulation of HeLa cells with the ability to take up and/or be killed by the toxic factor(s) present in HVSA, an attribute which could be dependent on either

phenotypic or genotypic heterogeneity. First, synchronous cultures of HeLa cells were prepared by mitotic selection and hydroxyurea treatment¹⁷ and used in the test system at four different time points spanning one growth cycle. No difference was detected in the susceptibility of cells to HVSA with respect to different phases of cell growth; in each case only 30% of the cells died. Second, attempts were made to select cells which exhibited a more uniform response to $MgSO_4$ stress when the latter is applied to cells in monolayers. This would be reflected in a steeper drop in the viability of cells beyond some critical concentration of $MgSO_4$. To date we have not succeeded in selecting a population possessing this kind of sensitivity as a stable characteristic. The third experiment was carried out with cells derived from the survivors of a toxicity test and again only 30% of the cells died. It is technically very difficult to carry out a quantitative test on the survivors. These experiments suggest that susceptibility to the cytotoxic factor depends on a hitherto undefined transient physiological state which allows some of the cells, on treatment with $MgSO_4$, to take up a lethal dose of cytotoxin.

In summary, we present evidence for the existence of vaccinia-specific protein(s) which under defined conditions can cause the death of susceptible HeLa cells. Formal proof that the toxic action is an intracellular as opposed to a membrane-mediated one is difficult to obtain. But no toxicity is induced by HSVA *per se* in contrast to, for example, adenovirus penton which interacts with cell membranes causing them to detach from glass surfaces¹.

Finally we would not extrapolate from these results to suggest that pox-virus infections are accompanied by a toxemia in the classical bacterial sense—no such evidence has ever been adduced—but we think that an examination of virus-specific products isolable from infected animal tissues would be worthwhile. This might lead to the recognition of virus-induced factors, which are toxic to the cells in which they are made, and which contribute to the overall complex mechanism of viral pathogenicity.

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Effects of dopamine-like drugs on rat striatal adenylyl cyclase have implications for CNS dopamine receptor topography

THE involvement of central dopaminergic neurones in the pathogenesis of several extrapyramidal movement disorders is well documented^{1,2}. Moreover it has been suggested that dopaminergic mechanisms may also be involved in the physiological mechanisms underlying psychoses³. For these reasons there is considerable neuropharmacological interest in the interaction between anti-Parkinsonian and neuroleptic drugs and central dopaminergic systems. Recently it has been shown that homogenates of tissues containing dopaminergic synapses respond to low concentrations of dopamine by an increased production of cyclic AMP. These areas include the bovine superior cervical ganglion⁴, the rat and bovine retina⁵, rat basal ganglia⁶, olfactory tubercle, nucleus accumbens⁷ and cortex⁸. The effects of dopamine in some of these systems are mimicked by dopamine receptor stimulating drugs such as apomorphine^{6,7} and 1-(3,4-dihydroxybenzyl)-4-(2-pyrimidyl) piperazine (S584)⁹ and antagonised by neuroleptic drugs^{6,10-12}. It seems, therefore, that these systems represent valid models of CNS dopamine receptors and it has been suggested that they may even comprise the dopamine receptor itself⁶. We have studied the dopamine-sensitive adenylyl cyclase of the rat striatum in order to define some of the structural requirements for dopamine receptor agonists.

In accordance with previous reports¹⁰, we found that addition of low concentrations of dopamine to rat striatal homogenates increased cyclic AMP accumulation during a

brief incubation *in vitro*. Half maximal stimulation was obtained at approximately 3 μ M dopamine. Experimental details are shown in Fig. 1. The effects of various compounds related to dopamine and apomorphine were also investigated. Homogenates were incubated with drugs at concentrations between 10^{-6} and 10^{-3} M. These results are summarised in Tables 1 and 2.

Dopamine and its N-methyl derivative epinine had similar activities as agonists. Further methyl group substitution of the amine function as in the N,N-dimethyl and the quaternary methyl dopamine derivatives led to compounds that were less active agonists.

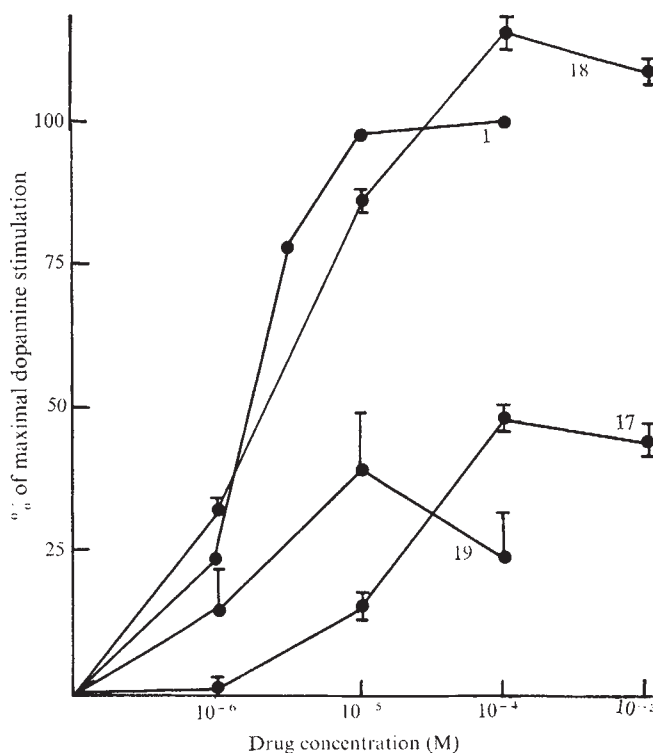


Fig. 1 Effect of cyclic analogues of dopamine on stimulation of cyclic AMP production in rat striatal homogenates. Sprague-Dawley rats weighing 180-250 g were used. The rats were decapitated and their brains rapidly removed and placed on ice. The striata were dissected as in ref. 25 and homogenised with a motor driven teflon/glass homogeniser in approximately 25 vol (w/v) of 2 mM Tris maleate buffer pH 7.4 containing 2 mM EGTA. Fifty microlitre aliquots of this homogenate were added to 250 μ l of 80 mM Tris maleate buffer, containing 2 mM $MgSO_4$, 0.2 mM EGTA and various drugs as indicated. The incubation tubes were kept on an ice/salt bath while ATP was added to a final concentration of 0.5 mM. The incubation tubes were then placed in a shaking water bath at 30°C for 2.5 min. At the end of this time the tubes were transferred to a boiling water bath for 2.5 min. The contents of each tube were centrifuged for 5 min in a microcentrifuge to sediment the denatured protein. 10 μ l aliquots of the supernatant were assayed for cyclic AMP content by the method of Brown *et al.*²⁶ using a linear standard curve produced with aliquots of authentic cyclic AMP between 0.2 and 8.0 pmol. The levels of cyclic AMP increased from a basal level of 31.8 ± 0.96 pmol per assay tube (approximately 2 mg wet weight tissue) to 66.9 ± 3.69 pmol per assay tube (mean value $\pm$ s.e.m. $n=9$) in the presence of a maximally stimulating concentration of dopamine (100 μ M). Incubations with dopamine at 100 μ M was included in every experiment for comparison. As the stimulation produced by 100 μ M was slightly variable between experiments the effect of each agonist was compared with the effect of 100 μ M dopamine in the same experiment using the same striatal homogenate. The effect of 100 μ M dopamine was normalised to 100%. Results are means of between four and 10 separate incubations, standard errors were less than $\pm 10\%$. Numbers on graphs refer to the structures given in Tables 1 and 2.

Table 1 Effect of phenylethylamine derivatives on cyclic AMP production in rat striatal homogenates

$ \begin{array}{c} R_3 \\ \\ R_1 - \text{C}_6\text{H}_3 - \text{X} - \text{N} - R_4 \\ \\ R_2 \end{array} $									
Name	R ₁	R ₂	X	R ₃	R ₄	R ₅	Max. Stim. (%)	† EC50M	
1 Dopamine	OH	OH	—(CH ₂) ₂ —	H	H	—	100	2 × 10 ⁻⁶	
2 Epinine	OH	OH	—(CH ₂) ₂ —	H	CH ₃	—	100	1.5 × 10 ⁻⁶	
3 N,N-Dimethyl dopamine	OH	OH	—(CH ₂) ₂ —	CH ₃	CH ₃	—	48	2 × 10 ⁻⁵	
4 N,N,N-Trimethyl dopamine	OH	OH	—(CH ₂) ₂ —	CH ₃	CH ₃	—	30	3 × 10 ⁻⁵	
5 <i>l</i> -Noradrenaline	OH	OH	—CH—CH ₂ —	H	H	CH ₃ +	97	4 × 10 ⁻⁵	
6 <i>dl</i> -α-Methyl dopamine	OH	OH	$ \begin{array}{c} \text{OH} \\ \\ \text{—CH}_2\text{—CH—} \\ \\ \text{CH}_3 \end{array} $	H	H	—	58	1 × 10 ⁻⁴	
7 <i>m</i> -Tyramine	H	OH	—(CH ₂) ₂ —	H	H	—	—	—	
8 <i>p</i> -Tyramine	OH	H	—(CH ₂) ₂ —	H	H	—	—	—	
9 3,4-Dihydroxybenzylamine	OH	OH	—CH ₂ —	H	H	—	—	—	
10 3,4-Dihydroxypropylamine	OH	OH	—(CH ₂) ₃ —	H	H	—	—	—	
11 3,4-Dihydroxybutylamine	OH	OH	—(CH ₂) ₄ —	H	H	—	—	—	
12 <i>d</i> -Noradrenaline	OH	OH	—CH—CH ₂ —	H	H	—	—	—	
13 <i>m</i> -Methoxytyramine	OH	CH ₃ O	$ \begin{array}{c} \text{OH} \\ \\ \text{—(CH}_2\text{)}_2\text{—} \\ \\ \text{CH}_2\text{—} \end{array} $	H	H	—	—	—	
14 <i>p</i> -Methoxytyramine	CH ₃ O	OH	—(CH ₂) ₂ —	H	H	—	—	—	
15 L-DOPA†	OH	OH	—CH ₂ —CH—	H	H	—	—	—	
16 <i>dl</i> -Amphetamine	H	H	$ \begin{array}{c} \text{COOH} \\ \\ \text{—CH}_2\text{—CH—} \\ \\ \text{CH}_3 \end{array} $	H	H	—	—	—	

* Stimulation produced by 100 μM dopamine is taken as 100%. For experimental details see Fig. 1.

† EC50 refers to concentration required to give half maximal stimulation produced between 10⁻⁶ and 10⁻³M. Agents shown as giving no stimulation of cyclic AMP production were not significantly effective (*P* > 0.05) at concentrations between 10⁻⁶ and 10⁻³M.

‡ Incubations included 0.4 mg ml⁻¹ of the dopa decarboxylase inhibitor NSD 1055 control incubations showed this had no effect on basal or dopamine-stimulated cyclic AMP production.

Compounds having 1–4 carbon atoms between the catechol nucleus and the primary amino group were examined. It was found that only dopamine, which has a 2 carbon side chain, was active. The analogues containing 1, 3 or 4 carbon atoms in the chain were all inactive. Incorporation of the side chain into a second ring system in an extended form as in 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene and apomorphine produced active agonists, whereas when the side chain was incorporated in a ring system in its nonextended form as in 6,7-dihydroxy-tetrahydroisoquinoline the resulting compound was much less active than the corresponding β-naphthylamine (Fig. 1). Substitution of a methyl group in the 1 position of the latter compound in salsolinol led to an inactive compound. Substitution of even larger groups at the 1 position also produced inactive compounds such as tetrahydropapaveroline and the emetic drug emetine.

Compounds lacking the catechol group were inactive. Thus the *m* and *p* isomers of tyramine, *m* and *p*-methoxydopamine, amphetamine, apomorphine, dimethoxy-apomorphine and 2-amino-1,2,3,4-tetrahydronaphthalene did not stimulate the adenylyl cyclase. Apomorphine, *dl*-α-methyl-dopamine and in particular 2-amino-6,7-dihydroxy 1,2,3,4-tetrahydronaphthalene, were all active in stimulating the enzyme system.

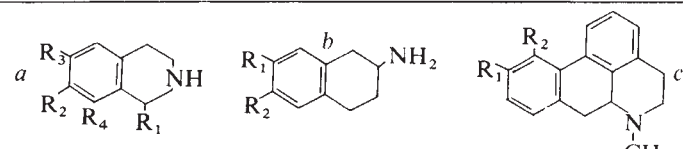
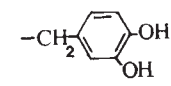
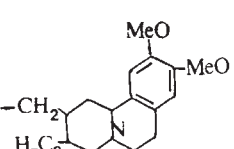
dl-α-Methyldopamine was less active than the parent compound. *l*-Noradrenaline was less than half as active as dopamine at a concentration of 1 × 10⁻⁵ M. The *d*-isomer of noradrenaline was inactive in concentrations up to 10⁻³ M. *l*-dopa was totally inactive.

As 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene was such a potent agonist, the effect of neuroleptic drugs on its adenylyl cyclase stimulating ability was examined. The clinically efficacious neuroleptic chlorpromazine (1 × 10⁻⁶ M) potently inhibited the stimulation of the

enzyme system by this agonist, whereas promethazine (1 × 10⁻⁵ M), which is a phenothiazine antihistamine with little or no neuroleptic activity¹³ was much less active (Fig. 2). Similar results have already been reported for dopamine⁷. In addition a maximally stimulating concentration of dopamine (100 μM) did not increase the stimulation produced by 100 μM 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene. These observations suggest that the two agonists have the same locus of action.

It is of interest that in two other dopamine-sensitive preparations, namely the renal artery of the dog¹⁴ and certain neurones of the snail *Helix aspersa*¹⁵, epinine was also found to be equipotent with dopamine. Among the simple dopamine analogues there is an absolute requirement for a catechol grouping and a 2 carbon side chain attached to an amino group. The structural and conformational requirements of the amino group are apparently less stringent, as even the quaternary methyl ammonium compound still retains some activity. Activity is also not abolished when the amino group is incorporated into a second ring as in 6,7-dihydroxy-tetrahydroisoquinoline. Due to the greater potency of the dopamine analogues where the side chain is incorporated in an extended form, as in 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene and apomorphine, it seems that the preferred conformation of dopamine on interaction with the adenylyl cyclase system is probably similar to the fully extended *trans* form. This has been shown to be the preferred conformation of dopamine in the solid state by X-ray crystallography¹⁶, in solution by NMR analysis and *in vacuo* by theoretical calculations¹⁷. Calculations based on published X-ray data for dopamine¹⁶ and apomorphine¹⁸ of the interatomic distance between the catechol oxygens O₁ and O₂ and the nitrogen atom of the amino group show that in dopamine N–O₁ is 6.83 Å, and N–O₂ is 7.83 Å. In the two *gauche* forms of dopamine

Table 2 Effect of β -naphthylamine, tetrahydroisoquinoline and aporphine analogues on rat striatal cyclic AMP production

Name	Basic Structure					Max* stim. %	EC50 M†
		R ₁	R ₂	R ₃	R ₄		
17 6,7-dihydroxytetrahydroisoquinoline	<i>a</i>	H	OH	OH	H	—	—
18 2-amino-6,7-dihydroxy-(1,2,3,4)-tetrahydronaphthalene	<i>b</i>	OH	OH	—	—	115	4×10^{-6}
19 Apomorphine	<i>c</i>	OH	OH	—	—	45	2×10^{-6}
20 Salsolinol	<i>a</i>	CH ₃	OH	OH	H	—	—
21 1-methyl-7,8-dihydroxytetrahydroisoquinoline	<i>a</i>	CH ₃	H	OH	OH	—	—
22 2-amino-1,2,3,4-tetrahydronaphthalene	<i>b</i>	H	H	—	—	—	—
23 Aporphine	<i>c</i>	H	H	—	—	—	—
24 Dimethoxyapomorphine	<i>c</i>	CH ₃ O	CH ₃ O	—	—	—	—
25 Tetrahydropapaveroline	<i>a</i>		OH	OH	H	—	—
26 Emetine	<i>a</i>		CH ₃ O	CH ₃ O	H	—	—

* Stimulation produced by 100 μ M dopamine is taken as 100%. For experimental details see Fig. 1.

† EC50 is taken as the concentration giving half maximal stimulation obtained between 10^{-3} and 10^{-6} M.

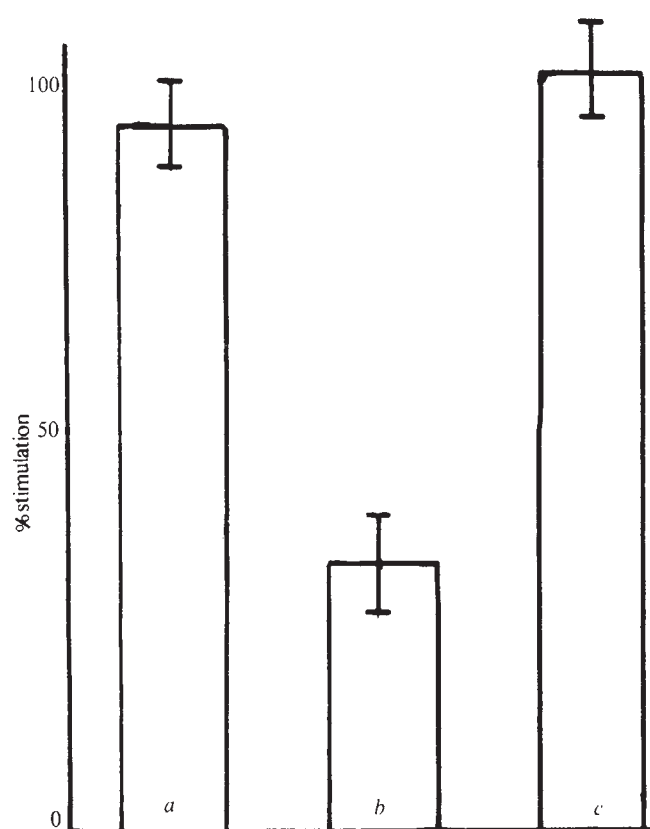


Fig. 2 Effect of *b*, 10^{-6} M chlorpromazine and *c*, 10^{-6} M promethazine on a maximally stimulating concentration of 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (10^{-4} M). *a*, 10^{-4} M 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene alone. Basal cyclic AMP levels for experiments in Fig. 2 were 32.5 pmol per assay tube (2 mg wet weight of tissue). For experimental details see Fig. 1.

(Fig. 3) these interatomic distances are significantly smaller¹⁹. This may be regarded as further evidence for the concept that the preferred active-site conformation of dopamine resembles the fully extended *trans* form. Molecular models of the rigid analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene clearly show that the conformation of the amino and catechol group is very similar to that occurring in the crystal structure of dopamine, thus probably accounting for its potency as an agonist. Similar suggestions have been advanced by Rekker *et al.*¹⁹ who have argued against Kier and Truitt's²⁰ suggestion that the *gauche* conformation of dopamine, and hence the tetrahydroisoquinoline portion of apomorphine, are the im-

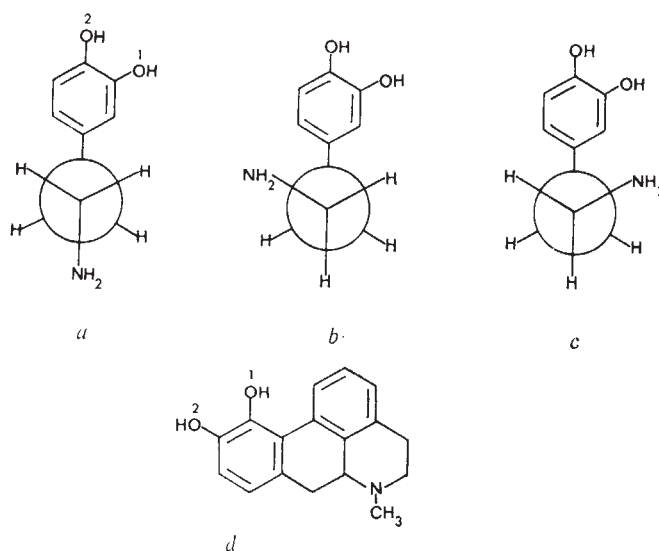


Fig. 3 *a-c*, Newman projections of dopamine conformers: *a*, *trans*; *b*, *c*, *gauche*; *d*, apomorphine.

portant conformations and moieties, respectively, for interaction with the dopamine receptor. The conclusions of Rekker *et al.* are supported by our present results. These findings also support the suggestion of Horn and Snyder²¹ that the preferred conformation of dopamine at its receptor site is similar to the fully extended *trans* form, and that this conformation could be superimposed on the X-ray structure of chlorpromazine, hence accounting possibly for the receptor blocking activity of the latter compound.

The results reported here may help in the design of future dopamine receptor stimulating agents. In particular rigid analogues of dopamine such as 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene or other similar compounds may be useful anti-Parkinsonian drugs^{22,23}. Initial studies suggest that 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene does not cross the blood brain barrier and possibly modifications of the molecule to increase its lipid solubility should be examined.

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Demonstration of indolaminergic fibres in the median eminence of the duck, rat and monkey

MANY reports provide physiological evidence that monoaminergic (catecholaminergic and serotonergic) systems are involved in the control of the secretion of the hypothalamic hormones (releasing factors) at the level of the median eminence (ME) of the rat^{1–3}. Dopaminergic and noradrenergic fibres have been shown in the median eminence by histofluorescence technique^{4–7}. This technique fails to demonstrate the serotonin (5-HT) fluorophores because of their low yield and rapid fading⁸.

As serotonergic neurones are able to take up and retain selectively endogenous or exogenous 5-HT, we have used ³H-5-HT to demonstrate by radioautography⁹ serotonergic, or more generally, indolaminergic neurones in the ME of three species; the duck (Pekin), the rat (Wistar) and the macaque (*Macaca mulatta*). Adult male ducks and rats and adult female monkeys were used as experimental animals. They were all pretreated with a monoamine oxidase inhibitor (nialmide) injected intraperitoneally at the rate of 200 mg kg⁻¹ for 4 h before administration of the tracer which was given either *in vivo* by intraventricular^{6,10} injection, or *in vitro* by incubation.

In the *in vivo* experiments, the animals were killed 30 min to 3 h after tracer administration. The animals were perfused with saline, then with 3.64% glutaraldehyde solution in 0.05 M phosphate buffer. The dissected tissues were postfixed in 2% OsO₄ in the same buffer, dehydrated in ethanol and embedded in Epon. For *in vitro* experiments, the following procedure was used. After decapitation, the median eminence was incubated in 5 ml of a physiological medium containing 0.2 μCi ml⁻¹ and 0.02 μg ml⁻¹ of ³H-5-HT, that is a 10⁻⁷ M concentration of ³H-5-HT. This low concentration was maintained by a continuous renewal of the incubation liquid. The incubation lasted 45 min at 37° C (ref. 11). The tissues were then rinsed in saline, fixed in glutaraldehyde, and treated for electron microscopy as described above.

In each case, radioautographic treatment was performed as follows: thick sections were placed on glass slides, then coated with K5 (Ilford) emulsion and exposed for 15 d before development in D19B (Kodak). Thin sections were put on celloidin-coated slides, stained with uranyl acetate and lead citrate and coated with a carbon film and a monolayer of L4 (Ilford) emulsion. After exposure for 3–6 weeks, the slides were developed in Microdol X (Kodak). The treated sections were transferred to copper grids and observed in an electron microscope¹².

In the three species, locations of radioautographic reactions after intraventricular injection of the tracer gave results identical to *in vitro* incubation. In the duck, very dense accumulations of silver grains were mainly localised in the most external layers of the ME in the vicinity of hypophysial primary portal vessels (Fig. 1). In the rat, clusters of silver grains were seen throughout the ME and were particularly abundant in the most external layers (Fig. 2a). In the monkey, the radioautographic reaction affected all layers of ME (Fig. 2b). Moreover, dense

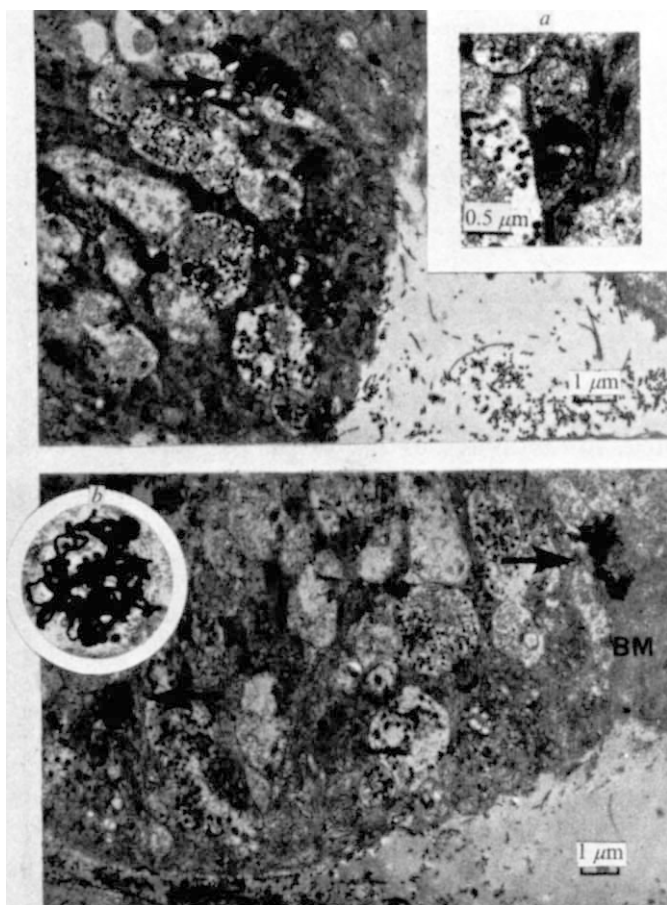


Fig. 1 Electron microscope radioautographs of two adjacent thin sections of duck ME after intraventricular injection of ^3H -5-HT. There are dense accumulations of silver grains on reactive fibres while the background is practically free. The comparison of the two pictures shows the labelling of the same fibre in the two sections. This fibre reaches the basement membrane (BM). Insets show *a*, a degenerating fibre in the same region after 5,6-DHT treatment and, *b*, an enlarged, labelled fibre with characteristic 1,000 Å dense granules.

and elongated clumps of silver grains frequently overlaid axonal bundles in the internal region.

In all three cases, electron microscopic examination revealed that labelled structures were axonal varicosities (from 0.3–1 µm) representing less than 1% of axonal sections in the ME. These labelled fibres contained densely packed inclusions: electron lucent vesicles and more scarce but very typical dense granules of larger size (1,000 Å) (Figs. 1 and 2). Some labelled terminals were seen in contact with the basement membrane close to portal vessels which are external and internal in mammals but only external in the duck. Other fibres made contact with a tanyocyte process but no synaptic differentiation could be seen at this level.

Our results give rise to three main questions concerning the nature of the radioactive molecules retained in the tissues, the identity of the labelled fibres and the function of such an innervation.

As the main pathway of catabolism of 5-HT is its oxidation by MAO (ref. 13), which was inhibited in our experiments, it seems probable that observed radioautographic reactions marked mainly the molecules of ^3H -5-HT.

The second question raises the problem of the specific labelling of indolaminergic neurones by ^3H -5-HT, as at high concentrations, 5-HT may be taken up into adrenergic neurones¹⁴. Thus, the most convincing evidence for selective uptake of ^3H -5-HT by 5-HT fibres comes from results of *in*

vitro incubations because at the concentration used (10^{-7} M), ^3H -5-HT is specifically taken up by serotonergic neurones¹⁵. Moreover, the ultrastructural features of the labelled fibres are clearly differentiated from those previously described in noradrenergic fibres^{6,7}. Noradrenergic fibres are larger and contain more scattered inclusions among which eccentric core vesicles appear to be characteristic. On the other hand, dense 1,000 Å granules seemed typical of ^3H -5-HT labelled fibres¹⁶. Finally, the pattern of labelled fibres after the administration of ^3H -5-HT was different from that obtained after administration of labelled catecholamines¹⁶. This fact was particularly clear in the duck ME where catecholaminergic fibres are concentrated in the internal zone.

A complementary pharmacological approach was attempted in this species to confirm the specificity of radioautographic results. Two intraventricular injections of 100 µg of 5,6-dihydroxytryptamine (5,6-DHT) which selectively destroys indolamine neurones^{8,17}, were administered 4 and 2 d before the application of the tracer. After this treatment, no incorporation of ^3H -5-HT was observed in the ME, and degenerating fibres appeared in the external zone (Fig. 1*a*).

All these results demonstrate the existence of indolaminergic fibres in the ME. To assume that they are truly serotonergic it would be necessary to identify them with structures containing 5-HT, the existence of which has been biochemically proved in bovine¹⁸ and duck¹⁹ ME.

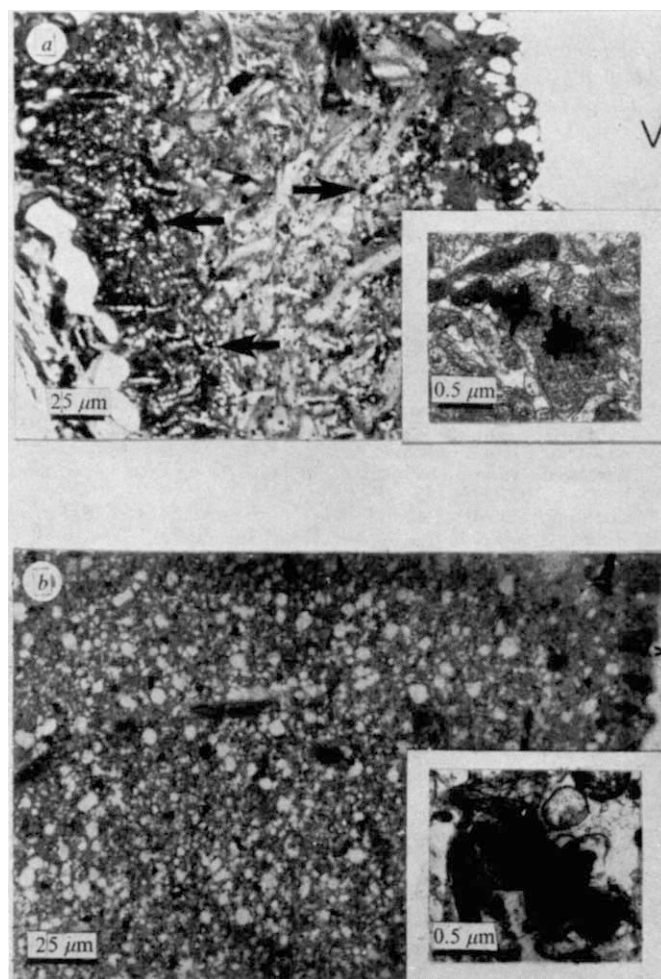


Fig. 2 Light microscope radioautographs of the ME of, *a*, the rat after intraventricular injection of ^3H -5-HT and, *b*, of the macaque after low concentration *in vitro* incubation in ^3H -5-HT. Note the localised distribution of radioautographic reaction and the low background. In the rat ME the tracer is preferentially localised near the internal and external plexuses. Insets show labelled fibres in each animal after low concentration *in vitro* incubation in ^3H -5-HT. V, Third ventricle.

In the absence of clearly defined synaptic junctions, we can only speculate on the function of the indolaminergic fibres²⁰. Their proximity to the processes of tanyocytes and to external basement membrane indicates that they may be involved in the transport of hypothalamic factors by glial cells and/or their release into the portal vessels.

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Antibody to bovine choline acetyltransferase and immunofluorescent localisation of the enzyme in neurones

THE neurones of the central nervous system that contain catecholamines, or serotonin, can be visualised histochemically by the highly fluorescent products that they form in the presence of formaldehyde vapour¹. No such elegant method exists for demonstrating the cholinergic neurones. Published methods for the histochemical localisation of cholinergic terminals and choline acetyltransferase (ChAc)^{2–5}, which is considered to be exclusive to them⁶, are indirect and non-specific. Recently, ChAc has been

purified and partially characterised from bovine caudate nuclei⁷. We report here (1) the production of specific precipitating antibody against bovine ChAc in the guinea pig; (2) characterisation of the antibody by immunodiffusion, by immunoelectrophoresis, by electrophoresis of the specific immunoprecipitates and by enzyme analyses, and (3) the visualisation of the cholinergic neurones in the central nervous system by the immunofluorescent method using specific antibody against purified ChAc. The specific antibody to ChAc can now be used in conjunction with the recently improved immunohistological technique⁸ to map the cholinergic pathway in the nervous system and to establish the localisation of the enzyme within the cholinergic neurone and its processes at the cellular level. Using the horse radish peroxidase technique⁸ and specific antibody to the glial fibrillary acidic protein, this astrocyte specific protein has been localised only within fibrous astrocytes and their processes at the light and electron microscopic levels⁹.

Specific precipitating antiserum to purified ChAc⁷ was prepared in albino male guinea pigs (600–700 g) as follows. Each animal received an emulsion containing 225 µg of ChAc, 0.7 ml 0.85% saline and 0.7 ml complete Freund's adjuvant (3.5 mg *Mycobacterium tuberculosis* H37RA) by intradermal injection distributed equally among twelve sites in the back. Seven days later an emulsion containing 125 µg ChAc, 0.7 ml 0.85% saline and 0.7 ml complete Freund's adjuvant was injected into six other sites in the back. Fourteen days after the first injection, an emulsion containing 125 µg ChAc, 0.7 ml of 0.85% saline and 0.7 ml of incomplete Freund's adjuvant was injected into six other sites in the back. Thirty-five days after the first injection, the two guinea pigs were bled by heart puncture.

Immunodiffusion and immunoelectrophoresis studies with the guinea pig antisera were performed as before¹⁰. Both antisera formed specific immunodiffusion lines against the crude extract of caudate nuclei, partially purified ChAc and purified ChAc but did not react against bovine serum or haemoglobin. The preimmunisation guinea pig sera did not react with any of the ChAc-containing extracts, bovine serum or haemoglobin. When the purified enzyme was subjected to immunoelectrophoresis at pH 8.6, one precipitin line was formed which corresponded to the area of migration for the ChAc when purified ChAc or a concentrated solution of crude extract of bovine caudate nuclei were used (Fig. 1).

Precipitates of immunoglobulin-ChAc complexes were prepared for electrophoresis by incubating the ChAc-containing solution with the antiserum for 1 h at 37°C followed by 20 h at 4°C. The precipitates were pelleted by centrifugation at 2,000g for 30 min in the cold and washed twice by resuspension in saline and centrifuged as before. Disc polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) of the crude enzyme extract, purified ChAc and immunoprecipitates were carried out as before^{10,11}.

The homogeneity of ChAc has been demonstrated by polyacrylamide gel electrophoresis at pH 9.5 and 7.3 at different gel concentrations (ref. 7). The enzyme behaved as only one sharp peak in sedimentation velocity studies (L.P.C. and F.W. unpublished results) and also only one peak was obtained on gel filtration (L.P.C. and F.W., submitted for publication). The multiple bands in the SDS-polyacrylamide gel electrophoresis are not contamination but are rather aggregated subunits of the enzyme since ChAc aggregates easily¹². Similar aggregation in SDS-polyacrylamide gel electrophoresis has been found for purified glutamic acid decarboxylase¹³, and this enzyme has been localised successfully in rat cerebellum by the immunohistochemical technique¹⁴.

Electrophoresis at pH 7 in SDS (Fig. 2) of the ChAc (gel 3a) showed a major component migrating in the range

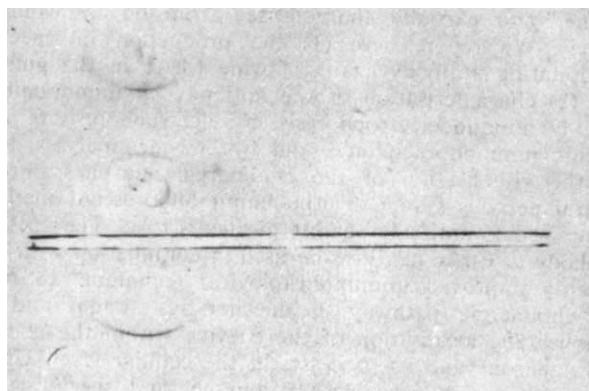


Fig. 1 Immunoelectrophoresis of purified bovine ChAc and crude extract of bovine caudate nuclei. Samples electrophoresed: *a*, ChAc; 3 mg ml⁻¹; *b*, crude extract of caudate nuclei, 17 mg ml⁻¹. Sera in the immunodiffusion troughs: 1, preimmunisation guinea pig serum; 2, serum containing specific antibody to ChAc.

of 60,000–67,000 molecular weight with additional minor components. Gels 2b and 2c are immunoprecipitates obtained by mixing the crude extract with the antiserum, and gels 3b and 3c are precipitates obtained by mixing the purified ChAc with the antiserum. In the absence of Cleland's reagent (reducing agent), the immunoprecipitates from the crude extract (gel 2b) and the purified ChAc (gel 2c) showed intact 160,000 molecular weight immunoglobulin and the ChAc bands. In the presence of Cleland's reagent, the immunoprecipitates from the crude extract (gel 3b) and the purified ChAc (gel 3c) showed the major enzyme band, the 53,000 molecular weight heavy chain, and the 25,000 molecular weight light chain of the dissociated immunoglobulin. The minor enzyme bands were obscured by the heavy chain immunoglobulin. The electrophoretic analyses of the specific immunoprecipitates demonstrate that the antiserum reacts specifically with the purified ChAc with was used to elicit the antibodies in the guinea pig.

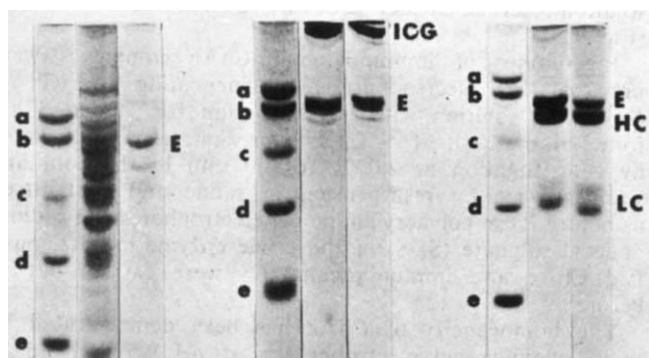


Fig. 2 Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of a crude extract of caudate nuclei, purified ChAc, and immunoglobulin-ChAc complexes. Gels 1a–3a contain 7.5% acrylamide. Gels 1b–3b and 1c–3c contain 10% acrylamide. The protein samples in gels 1a–3a and 1c–3c were treated with Cleland's reagent; gels 1b–3b were not treated with Cleland's reagent. Gels 1a, 1b and 1c were mixtures of conalbumin (*a*), serum albumin (*b*), ovalbumin (*c*), chymotrypsinogen A (*d*), and cytochrome (*e*). Gel 2a was the crude extract from caudate nuclei; gel 3a was the purified ChAc; gels 2b and 2c were immunoprecipitates obtained by incubating the crude extract with antiserum, and gels 3b and 3c were immunoprecipitates obtained by incubating the purified ChAc with antiserum.

For enzymatic studies, aliquots of partially purified ChAc (50 μ l) (which is more stable than the purified ChAc)⁷ were incubated with equal amounts of each of the three following solutions: (1) 0.05 M potassium phosphate, pH 7.0, containing 0.5 mM EDTA and 7% glycerol; (2) immunised guinea pig serum; (3) preimmunised guinea pig serum. The incubations were carried out at 37° C for 1 h and then at 4° C for about 20 h. The three mixtures were centrifuged at the end of incubation. The enzymatic activity in the supernatants and their corresponding resuspended precipitates were determined by the spectrophotometric method¹⁵ in the presence of creatinine-HCl¹². No precipitates were formed in the controls (mixture 3, preimmunised serum). White precipitates could be seen in the tubes containing ChAc alone (mixture 1) or ChAc plus immunised serum (mixture 2), however, the precipitates showed no enzyme activity. The enzymatic activity recovery was 88%, 69% and 95% in the supernatants for the ChAc incubated with solution 1, 2 and 3, respectively. About 30% of the ChAc was combined with its antibody during the incubation (solution 2) and this combined ChAc was inactive. There was only 59% recovery in the supernatant when the ratio of the ChAc to antiserum was 1:2 instead of 1:1. ChAc in the presence of preimmunised serum (solution 3) was more stable to prolonged incubation than in buffer alone (solution 1). This is consistent with our finding that other proteins have a stabilising effect on ChAc⁷.

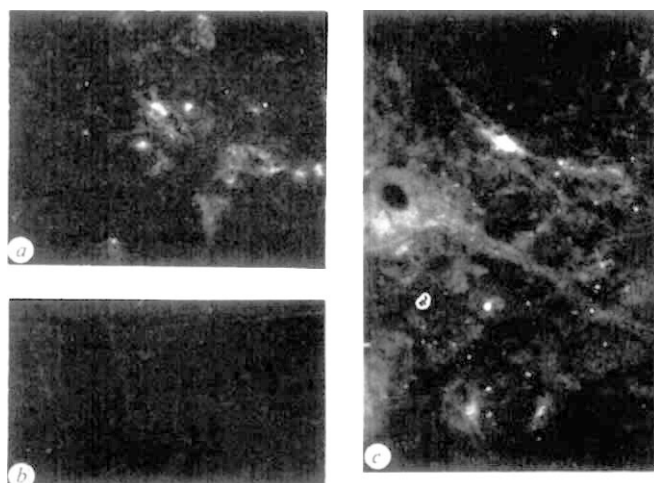


Fig. 3 Immunofluorescent localisation of choline acetyltransferase in 10 μ m frozen sections of the anterior horn of bovine spinal cord. *a*, Some of three anterior horn cells and their processes fluoresce while the adjacent white matter has only scattered spots of fluorescence. Note the absence of nuclear staining in the neurones ($\times 69$). *b*, Control anterior horn section with preimmunised guinea pig serum. *c*, Same as *a* ($\times 138$).

The anti-ChAc fluorescent antibody test was carried out by procedures previously reported¹⁰. Antiserum to guinea pig γ -globulin labelled with fluorescein was purchased from Antibodies Inc., Davis, California. Preimmunised and immunised guinea pig sera were used in 1:20 to 1:80 dilutions. Lyophilised cryostat sections (10 μ m) of the anterior horns of the lumbar enlargements of bovine spinal cord were extracted with chloroform for 15 s before incubation with antiserum. This removed the bulk of the lipid without affecting the activity of ChAc⁷. The cholinergic anterior horn cells were strongly fluorescent after incubation with guinea pig serum containing antibody to ChAc and then with the fluorescent goat antiserum (Fig. 3a). There was fluorescence also in the processes of the anterior horn cells but little in the neuropil and the adjacent white matter. Control sections using guinea pig serum before immunisation with ChAc were negative (Fig. 3b). Further studies

on the immunoenzymatic localisation of cholinergic neurones in the nervous system are in progress. While this work was in progress, antibodies to rat brain were reported¹⁶.

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Muscle membrane protein kinase in myotonic muscular dystrophy

MYOTONIC muscular dystrophy is an inherited disorder of man with manifestations in many organ systems including skeletal muscle myotonia and dystrophy, testicular atrophy, cataract formation, hypercatabolism of IgG, cranial bone malformations, and glucose intolerance with enhanced insulin levels (see ref. 1 for clinical references). Our previous experiments demonstrated a significant diminution in the phosphorylation of endogenous membrane proteins of frozen erythrocyte ghosts from myotonic patients¹. In subsequent studies the technique of electron magnetic resonance was employed to compare the erythrocyte membrane milieu of normal and myotonic patients². Following incorporation of 5-nitroxide methyl stearate into erythrocyte membranes, the spin label was located in a less polar and somewhat more fluid region in myotonic membranes than in normal membranes. Although such experiments do not specify the molecular membrane abnormality or its possible relationship to protein phosphorylation, they do support the suggestion that myotonic muscular dystrophy is a disease

resulting from a basic membrane abnormality.

Erythrocytes were initially chosen for our study because of the ease of purifying membranes from normal and myotonic patients. But more significant manifestations of the disease are in muscle where numerous physiological investigations have demonstrated alterations of the surface membrane³⁻⁶. The potentially low yield of surface membrane from biopsy specimens together with the numerous secondary changes which may be unrelated to the primary disease process retarded our early biochemical investigations of muscle membrane abnormalities. With the demonstration of protein phosphorylation in purified fractions of rat muscle membrane, the technical capacity presented itself for evaluation of human biopsy material⁷. In the data to be presented below, endogenous protein phosphorylation of muscle membranes was found to be diminished in myotonic muscular dystrophy compared to control tissue. These results confirm involvement of membrane protein phosphorylation and its usefulness as a sensitive index of membrane abnormality in myotonic dystrophy.

Six patients with myotonic muscular dystrophy representing six separate families participated in the study. All of these individuals gave informed consent. Each experiment involved one patient and one control. The control muscle was obtained from patients undergoing orthopaedic operations involving femur or hip repair. None of the controls had any known muscle disease. Quadriceps femori muscle was used in all cases. The control biopsies were obtained within 1 h of patient biopsy; and both tissue preparations were fractionated simultaneously at 4° C according to a modification of the method of Brody⁸. The resultant membrane pellet consisted of a mixture of sarcoplasmic reticulum, sarcolemma, and other membrane particles. Following determination of protein concentration⁹, assessment of endogenous protein kinase activity was carried out by a modification of the methods previously used in our studies. This incubation mix contained freshly prepared muscle membranes and 50 mM sodium acetate buffer (pH 6.5); the time of incubation was 20 s. All other reagents were identical to previous studies¹⁰. Following solubilisation the polypeptides were electrophoresed on 7 1/2% SDS polyacrylamide gels. Gel staining and determination of radioactivity were performed as previously described¹⁰. Na⁺, K⁺, Mg²⁺-ATP-ase of the membrane fractions¹¹, adenyl cyclase¹² and phosphoprotein phosphatase¹ were assayed.

Washed muscle membrane preparations from both control and myotonic tissues were able to incorporate ³²P into membrane protein using only γ -³²P-ATP as a substrate. As noted previously in erythrocytes and in rat muscle preparations both the enzyme and the protein substrate seem to be endogenous components of the membrane. In each experiment the rate of incorporation of ³²P was lower in the myotonic than the control membrane. In six experiments the total myotonic membrane phosphorylation averaged 64% of control activity; and this diminution is similar to that observed in erythrocytes (Table 1).

When the polypeptide profile of the membrane preparations from control and myotonic tissue were compared, no reproducible differences were noted. Three major components were found to be phosphorylated (Fig. 1). Phosphorylation of component 1 (molecular weight approximately 90,000-100,000) was variable and after repeated washing of the membrane preparation in the Tris sucrose buffer used in membrane preparation, both the amount of component 1 relative to the other peaks as well as the phosphorylation were considerably decreased. These data suggest that component 1 is either an exogenous cytoplasmic protein or is phosphorylated by a cytoplasmic enzyme which is contaminating this crude particulate fraction.

Components 2 and 3 were readily phosphorylated and were relatively unaffected by additional membrane washes. Component 2 had a greater apparent molecular weight than component 3 respectively as assessed by SDS polyacrylamide gel electrophoresis (50,000 compared with 30,000). Component

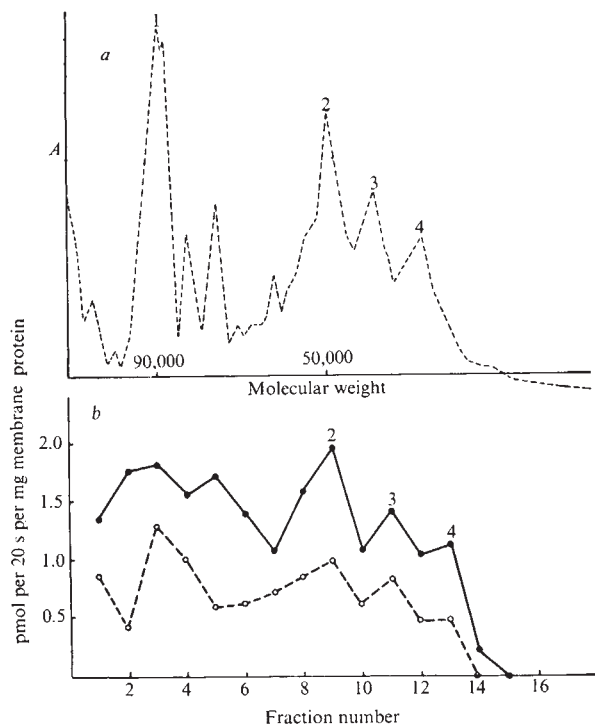


Fig. 1 *a*, Sodium dodecyl sulphate-polyacrylamide gel scan of a typical muscle membrane preparation. *b*, Radioactivity expressed as $\text{pmol per } 20 \text{ s per mg membrane protein}$ in SDS-polyacrylamide gel fractions. Experimental blanks consisting of boiled membrane or no Mg^{2+} have been subtracted. Results are from a typical experiment. ●, Control; ○, myotonic.

2 also had a slightly greater specific activity *in vitro* phosphorylation (Table 1). In all six experiments, the rate of incorporation of ^{32}P into components 2 and 3 was lower in myotonic membranes. In both components 2 and 3, the mean phosphorylation of myotonic membranes was 50% of control preparations.

Na, K (Mg) ATP-ase activities were identical in control and myotonic membrane fractions. Similarly basal, isoproterenol, and fluoride-stimulated adenyl cyclase activity demonstrated no reproducible differences (Table 2). Phosphoprotein phosphatase activity was absent in control and myotonic membranes under the conditions used in this study.

The major limitation to evaluation of enzymatic changes of membrane fractions of human muscle tissue is the limited yield of purified fractions which may be obtained from biopsy specimens. In most studies, investigators sacrifice purity for improved yield. But when doing so as in the present experiments, one should be extremely cautious about the extent to which similar membrane preparations are being examined in the control and diseased situations. The comparable polypeptide profile as assessed by polyacrylamide gel electrophoresis, the similar Na, K (Mg) ATP-ase activity, as well as hormone and

Table 2 Adenyl cyclase and ATPase in muscle membrane preparations

	Adenyl cyclase (pmol cyclic AMP per 10 min per μg protein)			ATPase ($\mu\text{mol P}$ per mg protein per h)	
	Basal	Isoproterenol (0.1 mM)	NaF Mg^{2+} (10 mM)	$\text{ATPase Mg}^{2+} + \text{Na}^+$	$+ \text{K}^+ \text{ATPase}$
Control	2.24	5.19	18.8	17.8	23.7
Myotonic	3.52	6.97	15.3	17.7	23.0

Data from a typical experiment. Adenyl cyclase assay was performed on fresh tissue. ATPase was measured on tissue frozen at -20° for 2 d. Each value is the mean of duplicates. In three additional experiments no differences in control or myotonic values were demonstrated, although each experiment resulted in slightly varying activities.

fluoride-stimulated adenyl cyclase all support the comparability of the membrane preparations. Nevertheless in animal experiments by Andrew *et al.* protein phosphorylation was found in a light density membrane fraction which could be separated from fractions containing the ATPase and cyclase^{7,13}. Thus our studies contrasting protein phosphorylation must be viewed with considerable caution because of the heterogeneous origins of the fractions used.

In rat muscle preparations no polypeptide larger than 30,000 was phosphorylated. In the present studies, phosphorylation of the 90,000–100,000 molecular weight component was variably affected in myotonic muscle preparations and could be altered in both normal and myotonic preparations by extensive washing. In rat muscle tissue phosphorylation of high molecular weight components is characteristic of cytoplasmic enzymes contaminating the membrane fraction. For this reason phosphorylation of this component was felt to be less significant than that of components 2 and 3.

Phosphorylation of both of these components was significantly diminished in myotonic muscle tissue. But in these muscle membranes, the altered activity cannot be definitely attributed to a diminished enzyme because comparable activity in control and myotonic membrane components 2 and 3 could be demonstrated when the pH was raised to 7.5 and assays were carried out in the presence of Tris buffer (Fig. 2). Similar

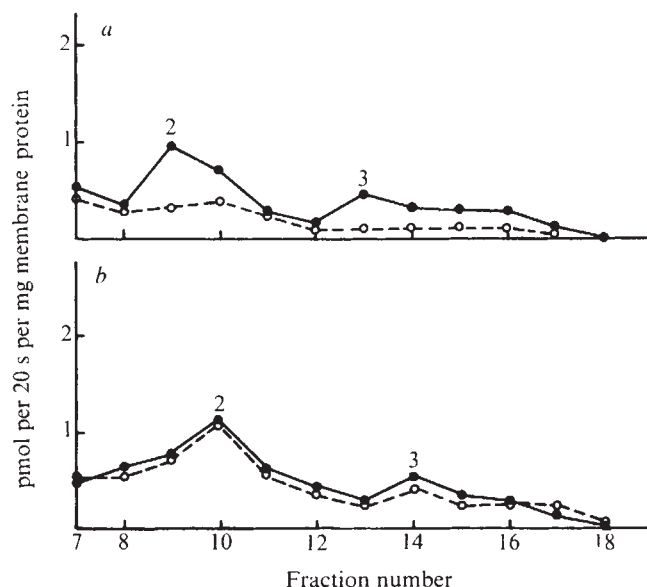


Fig. 2 *a*, Radioactivity expressed as $\text{pmol per } 20 \text{ s per mg membrane protein}$ in fractions corresponding to proteins 2 and 3 in a typical experiment using 10 mM Na acetate pH 6.5 as the buffer system. *b*, Same experiment performed using 10 mM Tris-HCl pH 7.5. ○, Control; ●, myotonic.

Table 1 Endogenous protein phosphorylation of muscle membranes

	Myotonic ($\text{pmol per mg per } 20 \text{ s}$)	Control ($\text{pmol per mg per } 20 \text{ s}$)	
Total membrane phosphorylation	9.95 ± 1.1	15.9 ± 1.8	$P < 0.02$
Phosphorylation of membrane component 2	0.818 ± 0.12	1.62 ± 0.21	$P < 0.01$
Phosphorylation of membrane component 3	0.545 ± 0.10	1.09 ± 0.15	$P < 0.025$

Data from all six experiments. Total membrane phosphorylation represents the sum of all protein components. All experiments were performed in duplicate with experimental blanks (boiled membranes or reactions with Mg^{2+} deleted). Values indicated are the mean $\pm$ s.e.m. P was determined by the Student's t test.

variations of protein phosphorylation with buffer, pH and method of membrane preparation have been observed with human erythrocytes. Since the enzymatic activity of protein phosphorylation within the membrane seems so easily affected by environmental changes, it seems more reasonable to ascribe the changes noted with myotonic muscle to an altered membrane rather than to a specific genetically mediated alteration in the protein kinase enzyme. The demonstration of lowered protein phosphorylation in muscle membranes confirms the similar observations made by us on erythrocyte ghosts. Although we were initially concerned that fibrosis, fatty infiltration, dystrophy or denervation might specifically affect the outcome, none of these seem to be particularly pertinent since morphological evaluation of the biopsies employed would suggest that minimal pathology was present in the muscle segments used. Whether the altered protein phosphorylation of muscle membrane and red blood cells or the physical alteration in the red cell membrane is the more pertinent aetiological factor is not known at present. Either of these alterations might be associated with an abnormal membrane, and both would lend support to our concept of myotonic muscular dystrophy as a diffuse disorder of membranes.

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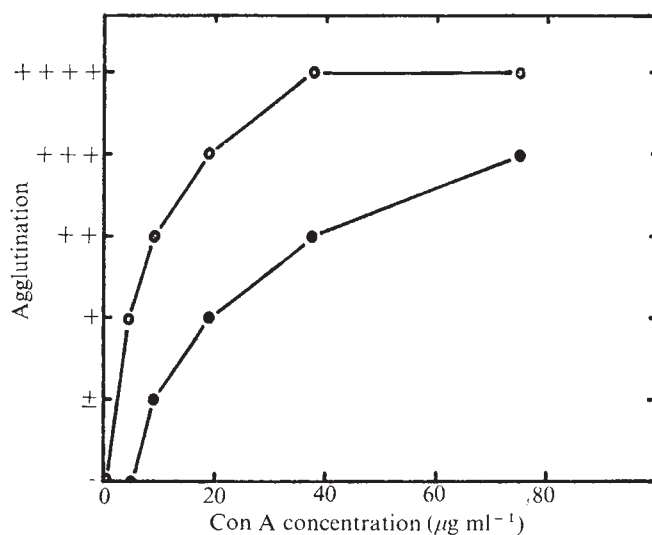


Fig. 1 Con A-induced cell agglutination of rabbit erythrocytes and reticulocytes. 10^8 cells were incubated with various concentrations of con A at 37°C for 20 min. Erythrocytes (●—●); reticulocytes (○—○).

changes on the membrane surface during the maturation of rabbit erythrocytes. We describe here the changes in cell agglutinability and in the binding sites of concanavalin A (con A) on the cell surface during erythrocyte maturation.

Reticulocytes were obtained from phenylhydrazine-injected rabbits⁶. Rabbit erythrocytes and reticulocytes were washed three times with glucose-free Hanks' balanced salt solution (HBSS). Con A and ^{131}I -labelled con A were obtained as described previously⁷. Cells were scored for agglutination on a qualitative scale from — to +++++ after 20 min incubation at varying temperature with an appropriate concentration of con A (ref. 7). The binding of ^{131}I -con A (672 c.p.m. per μg con A) was carried out at 37°C for 10 min with or without 100 mM α -methyl-D-glucoside (α -MG)⁷.

Reticulocytes agglutinated at much lower concentrations of con A at 37°C . Even at $5\mu\text{g ml}^{-1}$ con A, reticulocytes showed marked agglutination, whereas no agglutination was observed in the case of erythrocytes (Fig. 1).

The agglutination of both these cells was temperature dependent (Fig. 2). Erythrocyte agglutination was, however,

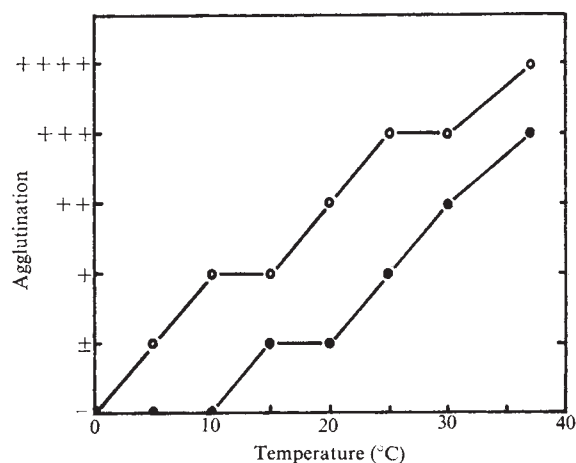


Fig. 2 Temperature-dependent agglutination of rabbit erythrocytes and reticulocytes. The concentration of con A used was $75\mu\text{g ml}^{-1}$. Other conditions as in Fig. 1. Erythrocytes (●—●); reticulocytes (○—○).

Structural difference in sites on surface membrane of mature and immature erythrocytes

MAMMALIAN cells transformed by oncogenic viruses or chemical carcinogens undergo characteristic changes in their surface properties such as lectin-induced cell agglutination^{1,2}. Normal cells show similar changes at their mitotic phase³ or after mild protease digestion of their surface membrane^{4,5}. This suggests that cells change their surface properties under various conditions and during the cell cycle. With this concept in mind, we studied the

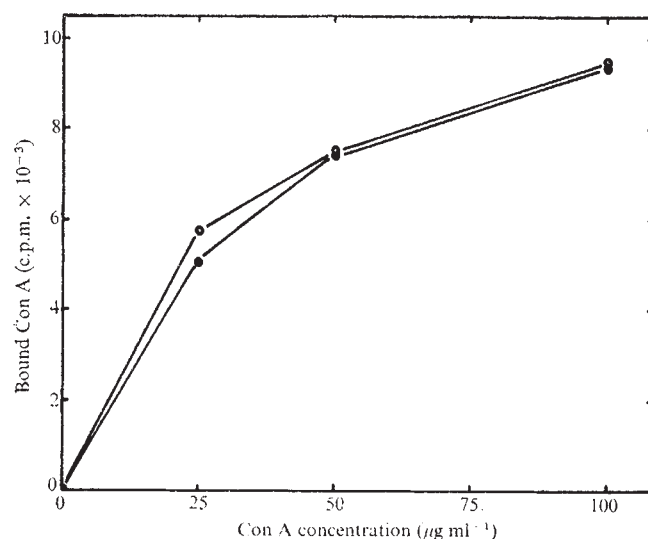


Fig. 3 Binding of ^{125}I -con A to rabbit erythrocytes and reticulocytes. 10^4 cells were incubated with varying concentration of ^{125}I -con A at 37°C for 10 min, with or without 100 mM α -methyl-D-glucoside, and were washed three times with HBSS. Erythrocytes (●—●); reticulocytes (○—○).

induced only at temperatures above 15°C whereas reticulocyte agglutination was observed even below 10°C . The difference in the agglutinability and in the temperature-dependence of these two cell types may be due to the difference in the number of surface receptors for con A.

The number of ^{125}I -con A binding per cell was, however, almost equal for each cell type (Fig. 3). This suggests that membrane changes other than the number of con A binding sites are likely to account for the difference in the agglutinability of these cells. The differences we have observed parallel the difference in the agglutinability of normal and transformed cells, and may be caused by the difference in membrane fluidity and/or topographical distribution of con A receptor sites on the cell surface. We are now doing a comparative study on surface structure and membrane components to understand the changes which occur during erythrocyte maturation.

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Isolation of a collagen-dependent cell attachment factor

Most untransformed mammalian cells require an appropriate surface for survival and growth *in vitro*; this phenomenon has been termed anchorage dependence¹. Glass, tissue culture plastics, fibrin clots and collagen surfaces have long been recognised as substrates able to support the attachment and growth of cells. Since the cell plasma membrane is separated from plastics substrates by a 450 Å layer of electron opaque material², the nature of the 450 Å would appear to be more important in cell attachment than the chemical composition of the plastic. It is demonstrated here that cell attachment to collagen is mediated by a high molecular weight protein present in serum.

Petri plates were coated with collagen as described in the legend to Fig. 1. When 2×10^5 SV-3T3 cells were added to collagenised Petri plates and incubated in the presence of serum for 1.5 h, the number of cells attached increased as the percentage of calf serum was increased from 0.03% to a plateau level at 3% (Fig. 2). Cell spreading was observed in the 10% and 3% points in 1.5 h. The sum of the attached and unattached cells (a) varied slightly with serum concentration and (b) was consistently slightly less than the number of cells inoculated (this may have been due to lysis of injured cells). By varying the time of the incubation, it can be seen that cell attachment started after 5 min, and continued for 6 h (at which time cell growth started in the high serum points) (Fig. 2a). The number of units of attachment factor, as defined in the legend to Fig. 1, varied as a linear function of time (Fig. 2b). The number of units of attachment factor did not vary greatly when the number of cells inoculated ranged from 1.5×10^6 to 2×10^6 (Fig. 3). It should be noted that the number of units of attachment factor varied between different commercial lots of serum.

The ionic requirements for cell attachment were determined by omitting components from Eagle's medium. Cells attached and spread in a medium consisting of NaCl, KCl (Mg^{2+} , or Ca^{2+}), glucose (at concentrations found in Eagle's medium) and 10 mM Hepes (pH 7.5) (no CO_2 or bicarbonate were used). The minimal attachment medium was 65% as effective as Eagle's medium as determined from the ratio of units of attachment activity in both media (omission of glucose or KCl results in less effective cell attachment). Cell attachment depended on either Mg^{2+} or Ca^{2+} , whereas Sr^{2+} and Ba^{2+} were inactive in the assay (Fig. 4). The pH optimum for cell attachment is in the physiological range, between pH 6.0 and 8.5 (high pH results in precipitation of Ca^{2+} and Mg^{2+} as their hydroxides).

The attachment factor was purified by batch preparation procedures as described in Table 1. The DEAE purified factor (Fig. 5) was half inactivated by treatment for 5 min at 65°C and had a pH of 4.8 (Fig. 6). Purified attachment factor (DEAE step) was stable to treatment at 37°C for 16 h with $10 \mu\text{g ml}^{-1}$ of either trypsin or pronase. The factor seemed to be of molecular weight greater than 200,000 since it was eluted at the void volume of both Sephadex G-100 and G-200 columns.

The rate of binding of attachment factor to collagen was assayed by (a) exposing air-dried collagenised plates to a series of dialysed cell attachment factor (second AmSO_4 step) concentrations in the presence of 0.9% NaCl (b) washing the plates eight times (2 min per wash) with 0.9% NaCl to remove unbound factor and (c) assaying cells in Eagle's medium without serum in order to determine the amount of factor that has been bound to the collagen. Figure 8 demonstrates that factor binds rapidly to collagen in the absence of divalent actions. Up to 30 min, the amount of factor bound increased linearly with time; the non-linearity in factor binding after 30 min may have been due to an increase in the number of collagen binding sites as the air-dried collagen swelled slightly during rehydration.

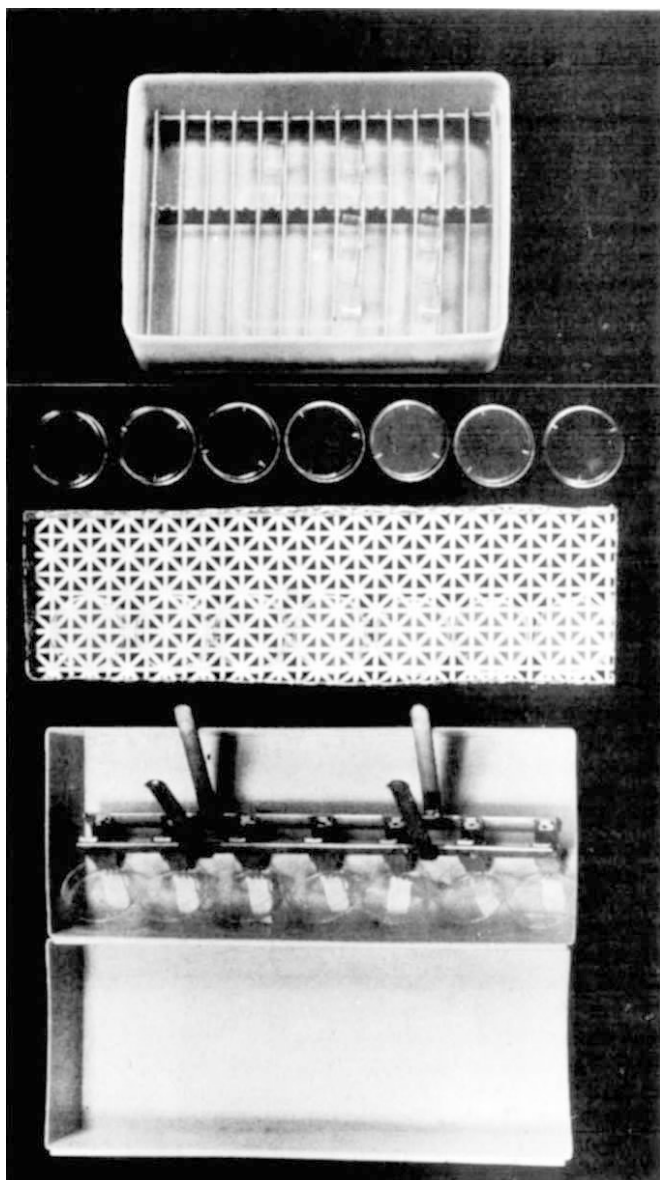


Fig. 1 Top, Petri plate washer, made from plastic box and bottom of a Rubbermaid (No. 6049) dish drainer; Middle, Petri plate tray; Bottom, Rubbermaid plastic boxes for saline and discarded solutions (used in washing Petri plates at the end of the assay); Bottom, Petri plate decanter, made from spring-loaded pants clamps and metal rod. Petri dishes were coated with collagen as follows. Collagen was prepared by acid extraction, and salt and ethanol precipitation^{4,6}. Freshly dissected rat tail tendons (8 g) were stirred overnight in 1 l of 0.2% citric acid and insoluble material was removed by filtration on a Buchner funnel. Collagen was precipitated by adding 10% (w/v) NaCl and was spun down at 10,000g for 15 min. The white precipitate was solubilised by stirring it in 0.2% acetic acid for several hours. The collagen solution was reprecipitated by adjusting the pH to 8.0 with NH_4OH , adding 20% (v/v) ethanol, and pelleting at 10,000g for 15 min. The collagen was lyophilysed and stored for up to 6 months at 15° C. Bacteriological Petri plates (Falcon No. 1007) were coated with collagen by placing the bases in a large plastic tray and dispensing into them 1.5 ml of 0.25% collagen in 0.2% acetic acid plus 0.002% phenol red with a 2-ml Cornwall pipetter. After rocking the tray to ensure uniform coating, the collagen was gelled by placing a partially NH_4OH -soaked, cloth-lined lid over the tray. The plates turned a metallic red colour in 5-10 min, when the lid was removed and the plates were air-dried. To reduce control levels in the assay, the completely air-dried plates were treated with 8 M urea for 20 min in Petri plate washing trays. The bottoms were washed three times with distilled water (5 min per wash) and then air-dried. The collagen-coated plates can be stored at room temperature for at least 2 months.

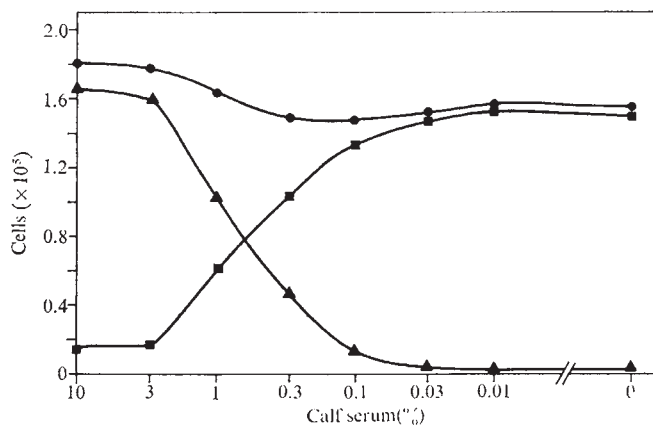


Fig. 2 Cell attachment assay. 2×10^5 cells were incubated at 37° C for 1.5 h on collagenised plates in the presence of serum. ▲, attached cells; ■, unattached cells; ●, attached + unattached cells. Petri plates were loaded with 5 ml of Eagle's minimal essential medium (Gibco catalogue No. F-11) containing $200 \mu\text{g ml}^{-1}$ bovine serum albumin (Sigma) with the required amount of serum or factor and preincubated at 37° C in a 5% CO_2 in-air incubator for 1 h (to allow the factor to bind to the collagen). Generally, serum concentrations in increments of one-half decade from 0.001% to 10% serum were used. During the Petri plate preincubation, semiconfluent SV-3T3 cells were (a) detached by a 15 min trypsinisation at 37° C with 0.25% Bacto-trypsin (Difco) and (b) washed twice in serum free Eagle's medium containing $200 \mu\text{g ml}^{-1}$ bovine serum albumin. After the Petri plate preincubation, 2×10^5 washed cells were added to each plate and incubated at 37° C in a CO_2 incubator for 1.5 h. Petri plates were emptied with the Petri plate decanter (Fig. 1), filled with 0.9% NaCl, decanted again and trypsinised for 10-15 min with 5 ml of 0.25% trypsin. The cells removed from the collagen were then counted with an electronic cell counter. Two samples of serum were compared with respect to cell attachment factor activity by (i) making serial half decade dilutions of the two samples and (ii) determining that percentage of serum which attaches 50% of the cells attached in the 10% serum controls. A unit of attachment factor is defined as the reciprocal of that percentage of serum that attaches half of the cells attached in the 10% serum controls (unit = $1/\% \text{ serum}_{1/2}$). The reciprocal of serum concentration is used to assign a higher number to a more active sample. The use of reciprocals does not change the magnitude of the ratio of activities, since $x/y = (1/y)/(1/x)$. As defined, the unit of attachment factor is proportional to the concentration of factor; the unit's dependence on time of incubation and number of cells used is given in the text.

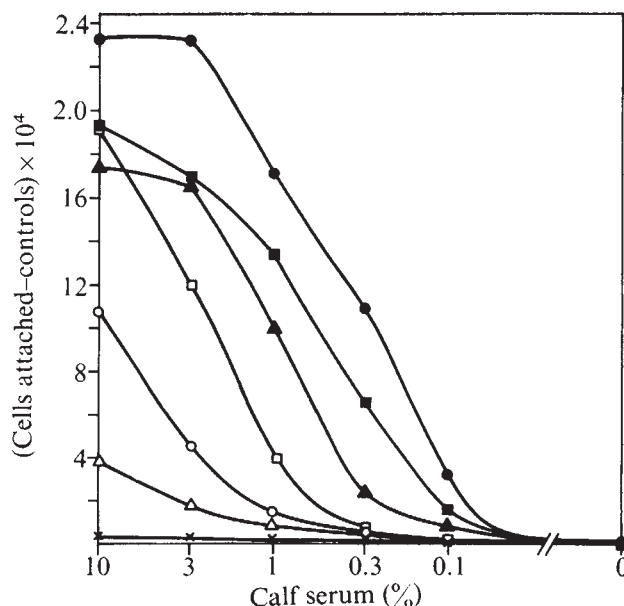


Fig. 3 Time course of cell attachment on collagen. ×, 5 min; Δ, 10 min; ○, 15 min; □, 30 min; ▲, 1.5 h; ■, 3 h; ●, 6 h.

The process of cell attachment to collagen can be divided into at least two steps. (1) A high molecular weight serum protein must bind to collagen; and (2) a divalent cation-dependent reaction is required for cells to attach to the collagen-serum factor complex. These steps can be distinguished from each other since the serum factor binds to collagen in the absence of divalent cations or cells; whereas, cells bind to factorised collagen only in the presence of Ca^{2+} or Mg^{2+} . Cell spreading on a collagen substratum may be mediated by the cell attachment factor, described here, since column fractions which mediate cell attachment also have cell spreading activity.

Collagen's role as a cell substratum may be identified as one of its major physiological functions from the following evidence. First, biochemical methods indicate that collagen represents 20% of total protein in mammalian tissues, with the exception of the nervous system³. Second, it is well known that collagen is found intracellularly in many tissues^{3,4} and is

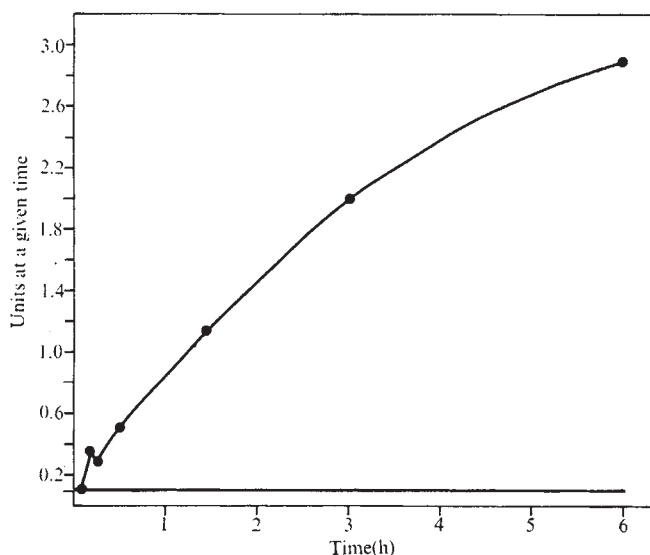


Fig. 4 Relationship between units of attachment factor and time (○).

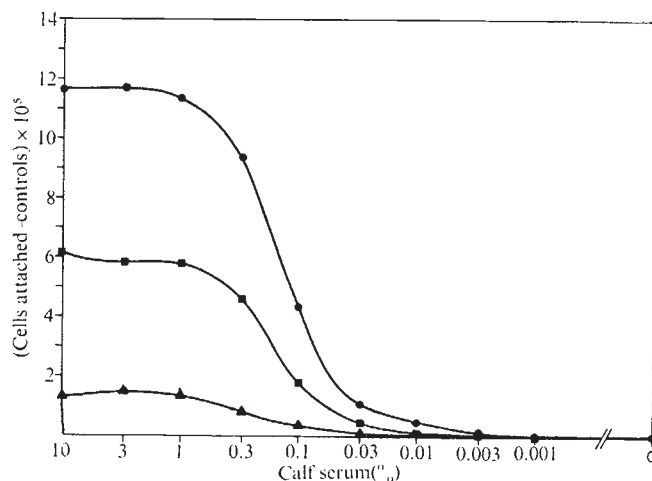


Fig. 5 2×10^5 (▲), 7.5×10^5 (■) and 1.5×10^6 (●) cells were incubated on factorised collagen for 3 h. More cells attached in low percentage serum when a high cell density was used than at high percentage serum when a low cell density was used.

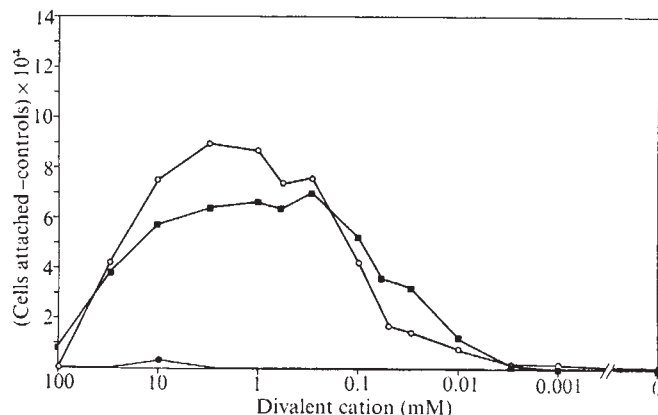


Fig. 6 Divalent cation requirement for cell attachment to collagen. 2×10^5 cells were incubated at 37°C in a medium consisting of NaCl; KCl; glucose (at Eagle's medium concentrations); 10 mM HEPES, pH 7.5; 10% calf serum equivalent of the DEAE purified factor (as assayed in complete Eagle's medium); and various divalent cations. No CO_2 was used to avoid the precipitation of carbonates. ○, Mg^{2+} ; ■, Ca^{2+} ; ●, Sr^{2+} or Ba^{2+} .

Table 1 Batch preparation of serum factor

Step	Total vol (ml)	Total protein (280nm; 1 cm path length) (fractions diluted to 1x calf serum)	Total units	Specific activity (units/280nm)	Recovery	Purification
Calf serum	1,000	71	14,300	201	—	1
1st PEG	200	20.16	14,300	711	100%	3.55
1st AmSO_4	200	8.4	12,500	1,488	87%	7.4
2nd PEG	200	5.4	10,000	1,851	70%	9.2
CaPi gel	400	2.2	1,940	882	13.6%*	4.4
2nd AmSO_4	50	1.32	3,300	2,500	23%	12.5
DEAE-11	90	0.30	2,500	8,350	17.5%	41.8

The serum attachment factor was purified from calf serum by batch preparation method. 1st PEG (0→4.5% PEG): using plastic labware to which PEG does not stick, 100 ml of a 50% (w/w) solution of polyethylene glycol-6000 (PEG) (Baker) solution was added to 1 l of calf serum at 4°C . The precipitate which had formed in 30 min was spun down at $18,000g$ for 30 min and dissolved in 200 ml of 0.1 M imidazole + 0.15 M NaCl (pH 6.8) (standard buffer). 1st AmSO_4 (0→28.6% AmSO_4): 80 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ was added slowly at 4°C to 200 ml of the 1st PEG material; after 20 min, the precipitate was collected by centrifugation at $18,000g$ for 15 min and dissolved in 200 ml of standard buffer. 2nd PEG (1→4.5% PEG): 4 ml of 50% PEG was added to 200 ml of the 1st AmSO_4 cut, and after 30 min the precipitate was removed by centrifugation at $18,000g$ for 10 min. 16 ml of 50% PEG was then added to the 1% PEG supernatant; after 30 min, the precipitate was collected by centrifugation at $18,000g$ for 20 min and dissolved in 200 ml of a standard buffer. CaPi gel: calcium phosphate gel (Brushite) was prepared according to Levin⁸. 200 ml of CaPi was added with stirring to 200 ml of the 2nd PEG material at room temperature. After 30 min, the gel was (i) spun down at $5,000g$ for 10 min; (ii) resuspended in 200 ml of 0.1 M aspartate (pH 6.8); (iii) spun down at $5,000g$ for 10 min, and (iv) eluted twice with 200 ml of 0.5 M aspartate + 0.1 M imidazole + 0.15 M NaCl (pH 6.8). 2nd AmSO_4 (0→30% AmSO_4): to the 400 ml of combined eluate was added 320 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ at 4°C ; the resulting precipitate was spun down at $18,000g$ for 15 min, and redissolved in standard buffer to a final volume of 50 ml. DEAE-11 chromatography: 25 ml of the 2nd AmSO_4 material was diluted to 100 ml with cold water and precipitate which had formed in 30 min was removed at $10,000g$ for 10 min. The soluble material was then applied to a 4.5×20 cm DEAE-11 column which had been washed with 0.5 M KH_2PO_4 (pH 6.8) and 200 ml of one-quarter strength standard buffer. The column was then washed with 60 ml of 0.25 M standard buffer. The active material was eluted with 1 l of 0.25 M NaCl + 0.1 M imidazole (pH 6.8) and the active peak was pooled (Fig. 5).

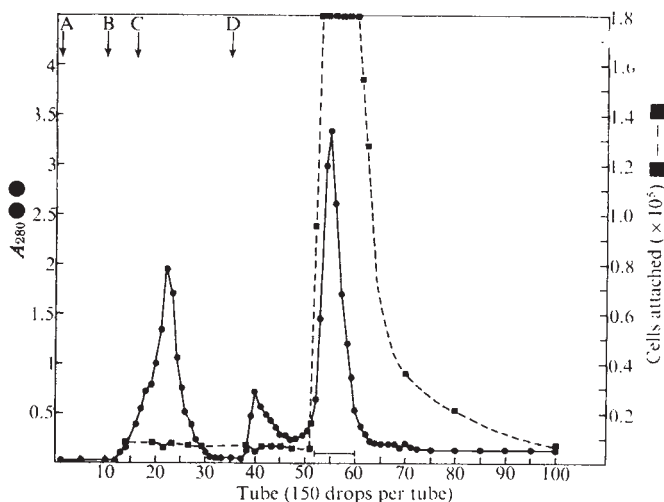


Fig. 7 DEAE-11 chromatography of 2nd AmSO_4 fraction. At each arrow, the following solutions were added to the 4.5×20 cm column: (A) 100 ml of diluted factor; (B) 60 ml of 0.25 strength standard buffer; (C) 200 ml of 0.65 strength standard buffer; (D) elution with 1 l of 0.25 M NaCl + 0.1 M imidazole (pH 6.8). Tubes 52 to 60 were pooled.

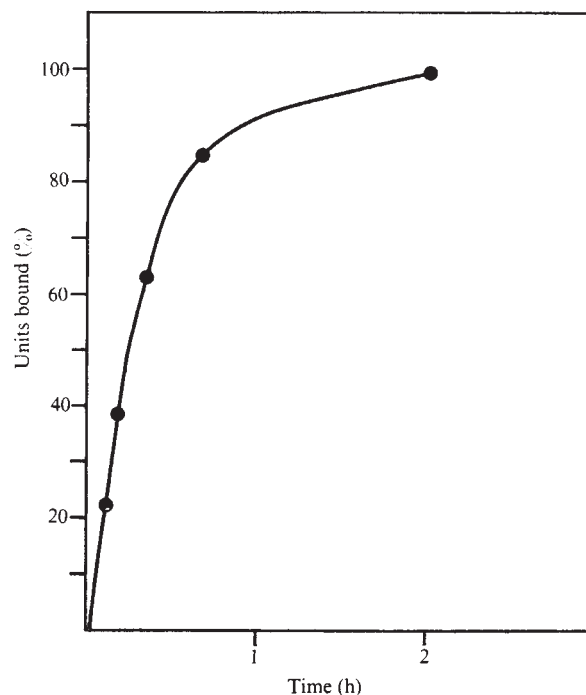


Fig. 9 Binding of purified attachment factor to collagen in the absence of divalent cations or cells. Collagenised plates were treated with a series of dialysed factor concentrations in 0.9% NaCl and treated as described in the text. The washed plates were assayed for attachment activity in Eagle's medium (with Ca^{2+} and Mg^{2+} + 200 $\mu\text{g ml}^{-1}$ bovine serum albumin).

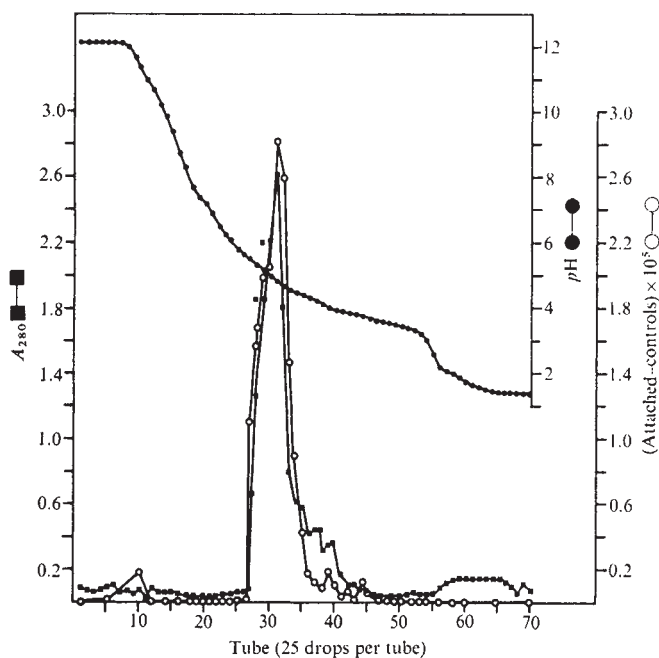


Fig. 8 Isoelectric focused in a LKB 110 ml column, in a sucrose gradient containing 1.4 ml pH 3-6 LKB ampholytes and 0.14 ml pH 3-10 LKB ampholytes.

produced by most tissue culture cell lines⁵. Finally, cell attachment to collagen has been shown here to depend on physiological parameters; namely, a requirement for divalent cations as well as a specific high molecular weight protein.

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Effect of zinc on haemoglobin binding by red blood cell membranes

RESULTS of recent studies in our laboratory have suggested that zinc plays an important role in sickle cell anaemia. A significant proportion of sickle cell patients are zinc deficient¹. Zinc binds to haemoglobin and increases oxygen affinity^{2,3}. Sickle cells treated *in vitro* with zinc show markedly improved filterability at concentrations too low to be explained on an oxygen affinity basis⁴, and zinc may interact with the membranes to affect filterability. A recent report suggests that sickling involves the accumulation of calcium⁵ which is known to reduce red cell membrane deformability⁶. We have now found that zinc decreases the amount of haemoglobin associated with red cell membranes and inhibits the effect of calcium in causing haemoglobin retention by membranes.

Single stage red cell membranes were prepared by the technique of Hoffman⁷ with minor modifications. Agents to be incorporated into these membranes were added during

Table 1 Haemoglobin retention by ghost cells

Sample type	Hb (g% in initial packed cells)	Hb (g% in final ghost pellet)	Mean cell volume (μm^3)	Hb (g% corrected for change in MCV)	% Retention of Hb	t test
No addition (control)	25.21 $\pm$ 1.8	1.86 $\pm$ 0.6	73.6 $\pm$ 1.9	1.86 $\pm$ 0.6	7.4 $\pm$ 2.5	$t = 6.3$ $P < 0.001$
ZnSO ₄ ghosts	24.94 $\pm$ 0.8	0.79 $\pm$ 0.2	22.9 $\pm$ 2.5	0.25 $\pm$ 0.1	1.0 $\pm$ 0.3	
CaCl ₂ ghosts	25.81 $\pm$ 1.0	21.79 $\pm$ 2.4	21.5 $\pm$ 1.6	6.39 $\pm$ 0.9	24.6 $\pm$ 4.1	$t = 4.5$ $P < 0.01$
CaCl ₂ + ZnSO ₄ ghosts	24.48 $\pm$ 2.1	10.41 $\pm$ 4.0	22.1 $\pm$ 2.3	2.50 $\pm$ 0.5	10.7 $\pm$ 2.6	

All values are the mean $\pm$ 1 s.d. of four observations. ZnCl₂ had approximately the same effects when substituted for ZnSO₄.

haemolysis. The principle of this technique is based on the knowledge that erythrocyte membranes lose their selective permeability at haemolysis and immediately thereafter, facilitating the introduction of various normally nonpenetrating compounds into the cells^{8,9}. In our experiments normal red cells to be haemolysed were divided into four aliquots and treated as follows: no addition (control), zinc sulphate (1.5 mM), calcium chloride (1 mM), and calcium chloride (1 mM) plus zinc sulphate (1.5 mM). Hypotonic exposure lasted 20 min. In all but the control aliquot enough sodium chloride was added to achieve the same ionic strength as the calcium chloride plus zinc sulphate solution. After hypotonic exposure with 10 volumes of water the red cell membranes were resealed with isotonic saline—0.01 M Tris buffer, pH 7.4, and washed until the supernatant was clear of haemoglobin. The haemoglobin in these ghost cell preparations was measured by the cyanmethaemoglobin method. The size and the mean cell volume (MCV) of the ghost cells were calculated using a Coulter counter with multichannel particle size analyser and recorder. Haemoglobin concentrations in the membrane preparations were corrected for the decreased size of the red cell membranes compared with original red cells, since shrinkage of these ghost cells results in some increase in concentration of entrapped haemoglobin.

Table 1 shows that the amount of haemoglobin retained in the membrane preparations after final washing was much less in the presence of zinc compared with control membranes. In the presence of calcium a large amount of haemoglobin was retained, as reported before^{10,11}. When zinc was present together with calcium, however, much less haemoglobin was retained compared with calcium alone, although in both cases the membranes were similar in size (both were small). Equimolar concentrations of lanthanum chloride, but not magnesium, also decreased the haemoglobin-retaining effect of calcium in these preparations (data not shown).

Calcium is known to interact with the interior of the red cell membrane, altering its configuration, decreasing passive permeability¹⁰, decreasing deformability^{6,11} and increasing haemoglobin retention^{10,11}. Zinc seems to counteract the retention of haemoglobin by red cell membrane both in the presence and absence of added calcium. One effect of calcium may be to crosslink haemoglobin and membrane sites. If so, calcium may be involved in the pathogenesis of irreversibly sickled cells by promoting such crosslinking. It is tempting to speculate that zinc improves the filterability of sickle cells, and promotes the elution of haemoglobin from red cell membranes, by blocking our hypothesised calcium-induced crosslinking of haemoglobin to membranes.

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Nuclear segregation in *Bacillus subtilis*

JACOB, BRENNER AND CUZIN¹ suggested that prokaryotic nuclear segregation was achieved by cell surface extension between surface sites to which the chromosome is permanently attached (Fig. 1a). Their model (model A) showed nuclear segregation occurring in newly divided cells, with all surface extension in one cycle occurring between the nuclei. Thus at cell division, nuclei would be arranged symmetrically in the half cell but asymmetrically during the interdivisional period. If nuclear segregation occurs during mid-cycle and cell extension is continuous throughout the cycle, the nucleus cannot reach a symmetrical position (that is at 25% of the cell length) by the end of the cycle relying solely on length extension. To overcome these difficulties, Clark² proposed that the nucleus is always located at the junction of old and new membranes with growth occurring on one side of the nucleus (model B, Fig. 1b). At nuclear segregation, the growing point divides allowing growth of the cell envelope between the nuclei as shown in Fig. 1b. Nuclei remain attached at the site of envelope extension, giving a newborn cell with an asymmetrically arranged nucleus. Donachie and Begg³ have presented evidence for terminal growth regions in slow growing cells as predicted by Clark's model. A third possibility is that the nucleus is located centrally in new born cells and moves to a position at the centre of a half cell at the time of segregation (model C, Fig. 1c). These, and other possibilities, can be explored by determining the position of nuclei in relation to cell length in exponential phase cells.

Bacillus subtilis (168/S) (an asporogenic derivative of 168 *tryp⁻ thy⁻* able to grow on succinate as sole carbon

source at a generation time of 115 min) was grown in either succinate or glucose based minimal medium to minimise the cell separation time⁵ and to give substantial numbers of mononucleate cells⁴. Using Giemsa, nuclei appeared as small spherical dark blue bodies in a pale pink-to-colourless cytoplasm. The number and appearance of nuclei was highly reproducible under constant cultural conditions. Cells contain an average of 1.3 and 1.8 nuclei per unit on succinate and glucose media respectively. Each bacterial particle has been regarded as a unit even while containing a septum. The time interval between nuclear segregation and cell separation, calculated from the age distribution theorem⁵ is 48.5 and 45 min for succinate and glucose (generation times 115 and 57 min). Large numbers of cells were photographed and, from the negatives projected on to a screen, positions and diameters of nuclei and lengths of cells were determined. Less than 5% of any population could not be scored because of poor staining and a slightly larger proportion was discarded that were not in focus. All measurements of the position of nuclei were taken from the distal edge of the nucleus to the centre of the cell. These data are shown in Fig. 2.

The critical difference between model C and the other two is the abrupt transition of the nucleus from the centre of the cell to the centre of the half cell. If nuclei move apart gradually at early stages of segregation the nuclear attachment sites should be sufficiently close that nuclei would overlap and be scored as one larger than average nucleus. When plotted against cell length, the distance between cell centre and the distal edge of the nucleus should vary continuously as the two nuclei become visible. Figure 2 indicates that this is not so, and in fact the distal edges of nuclei of 85% of binucleate cells are at least 2.5 radii from the cell centre. Nuclei vary only slightly in size between mononucleate and binucleate cells. The best line connecting binucleate and mononucleate cells has a slope of about 1.5 in both media indicating that the nucleus moves along the cell apparently three times faster than the rate of length extension. The frequency distribution of distances from the cell centre to centre of nuclei are shown for two size classes of binucleate cells in Fig. 3. The peak for all cells is between 20 and 24% from the centre, but there is a skew towards the centre in smaller cells. The data are shown relative to the average nuclear radius of mononucleate cells and there is only minimal overlap between mono and binucleate cells. The peak of the distribution of distance from the cell centre is slightly less than 25%, possibly because

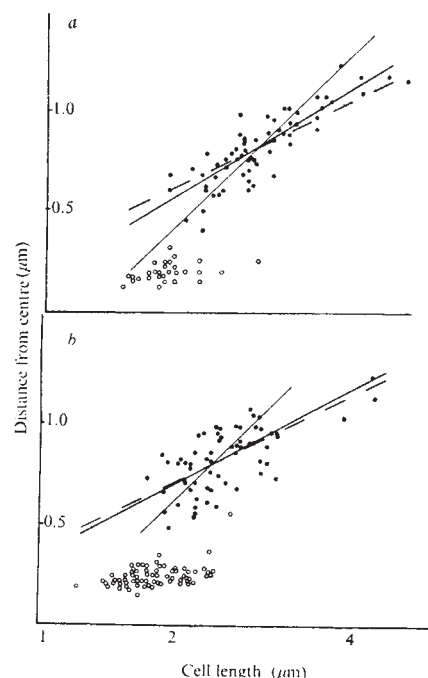


Fig. 2 Position of nuclei relative to cell length. *a*, Glucose grown cells (96 units in sample); *b*, Succinate grown cells (247 units in sample). To stain the nuclei, bacteria were heat fixed onto glass slides, treated with 1 N hydrochloric acid at 60° C for 7 min, washed in phosphate buffer (pH 7.4), and stained with Giemsa (diluted 1:10 with distilled water) for 5 min. ●, Binucleate cells; ○, mononucleate cells; ---, graph of 25% of cell length; —, graph of 50% of cell length; —, line of best fit (obtained by method of least squares) for distal edge of nucleus. Regression coefficients for the glucose grown cells (*a*) for distal edge and nuclear centre respectively were 0.31 (significantly different to 0.25 at 1% probability using Student's *t* test) and 0.27 (not significantly different at 5% probability) respectively. The correlation coefficients were 0.87 and 0.76 respectively. Regression coefficients for the succinate grown cells (*b*) for distal edge and nuclear centre respectively were 0.25 and 0.23 and were not significantly different to 0.25 at 5% probability. The correlation coefficients were 0.67 and 0.65 respectively.

cell length has been systematically slightly overestimated. As less than 5% of mononucleate cells show any deviation from the centre of the cell, the nuclei are probably located at 25% from the cell centre immediately before cell division.

Model B proposes that nuclei should be arranged asymmetrically in most cells of an exponential population. Less than 5% of mononucleate cells show any asymmetry on either medium, and the data in Fig. 3 indicate that nuclei of most binucleate cells are arranged symmetrically in each half cell.

Model C differs from A and B in the slope of the line relating nuclear position with cell length. Models A and B predict a slope of 0.5 whereas model C predicts a slope of 0.25. Although for both sets of data the scatter is considerable, the data favour model C rather than A or B (Fig. 1). Using autoradiography, Mendelson⁶ has shown that nuclei in binucleate cells of *Bacillus subtilis* 168 are predominantly 25% from either pole but mononucleate cells were not included in his analysis.

The rapid transition (less than 6 min) indicated by these data may be illusory, as in growing cells the nucleus may be arranged more diffusely than stained preparations suggest. If so, the location of the fixed nucleus probably represents the focus of the chromosomes' strongest interactions with the cell surface. The nucleus, however, may form a loose link with a new site earlier in the cell cycle. In *Escherichia coli*, segregation occurs close to the time of

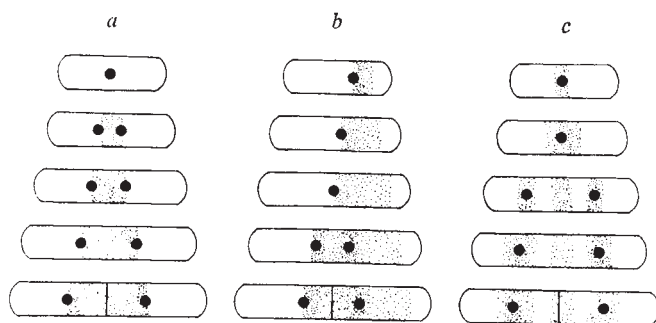


Fig. 1 Models of nuclear segregation in bacteria. *a*, The model of Jacob, Brenner and Cuzins¹ (model A). All surface growth occurs between the nuclei. *b*, The model of Clark² (model B) in which nuclei are rigidly attached to a particular site and growth occurs only to one side of the nucleus. *c*, An alternative model (model C) in which nuclei move to a site 25% of the cell length from the poles. Surface growth occurs to both sides of the nucleus. Most intense stippling indicates newest cell envelope.

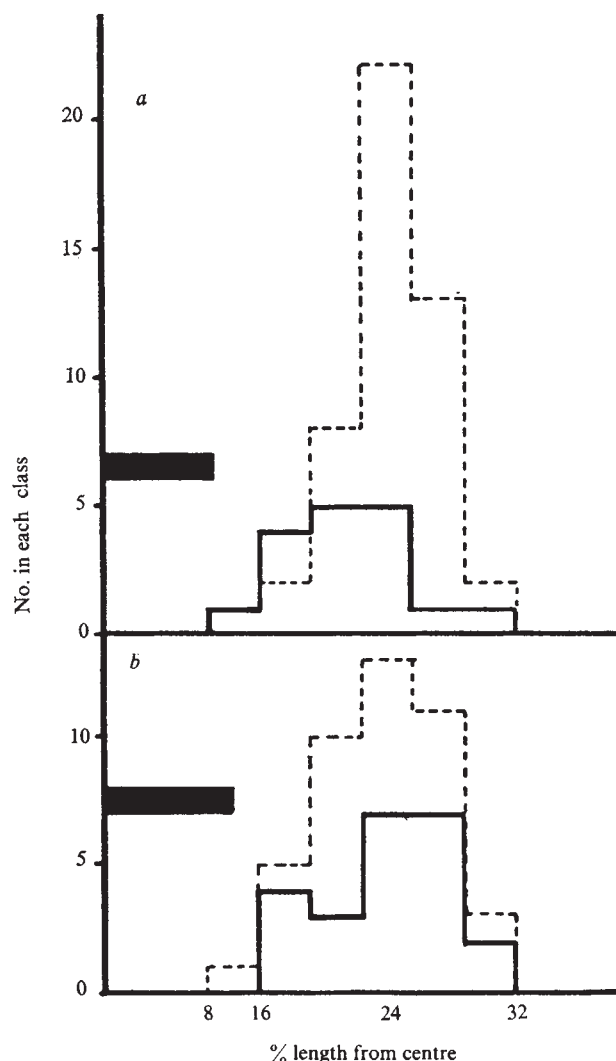


Fig. 3 Frequency distribution of distances from cell centre to centre of nuclei. *a*, Data from Fig. 2*a*. —, Data from binucleate cells less than 2.8 μm long. ---, Cells of greater length. *b*, Data from Fig. 2*b*. —, Binucleate cells less than 2.6 μm long. ---, Cells of greater length.

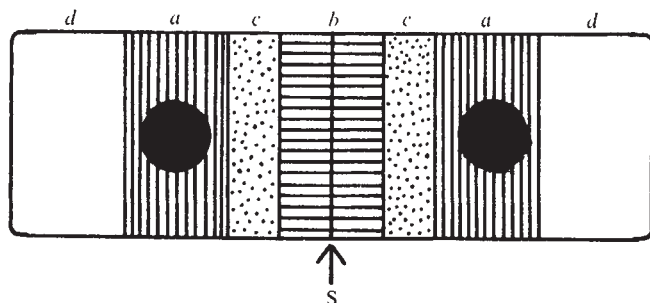


Fig. 4 Postulated origin of cell surface for a cell at division. Length of zone calculated assuming linear rate of surface extension from sites occupied by nuclei and that segregation occurs at mid-cycle (S, septum). *a*, Synthesised in interval between segregation and cell division; *b*, synthesised before segregation in current cycle; *c*, synthesised in interval between segregation and cell division in previous cycle; *d*, synthesised before segregation in previous cycle.

chromosome termination⁷ and this may represent the signal for pre-existing weak interactions with new sites to become predominant.

If the site of nuclear attachment is a major site of cell envelope extension and the rate of length extension is linear at each growth zone, the pattern of surface growth shown in Fig. 4 follows from the pattern of nuclear segregation in model C. The diagram shows the postulated distribution of cell surface synthesised during four periods for a cell which is about to divide. Lengthwise growth between segregations (Fig. 4*b* and *c*) must equal half the length of a cell at segregation. Therefore, if nuclei move 25% of the cell length, they would move to the junction of old and new surface formed one generation previously. As this was the site occupied by the nucleus at the time of the previous segregation, 'the pre-existing weak interactions', discussed here, may have been established at this time.

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Chronic response of dogs to parathyroid hormone infusion

WHEN parathyroid hormone is infused chronically at a near-physiological rate, it causes hypercalcaemia by a mechanism fundamentally different from that which follows acute injections. Hormones, like drugs, have characteristic rates of metabolism and excretion and their distribution in the body is affected by binding to plasma proteins and membranes. Thus similar dependence of the pattern of response on entry rate is to be expected in the case of other hormones with short half lives, acting on multiple receptors of varying sensitivity.

As discussed in a recent review¹, parathyroid hormone (PTH) is an 84-residue single-chain polypeptide, which is probably secreted continuously at a rate regulated by the calcium concentration of the plasma. Its principal function seems to be to raise the calcium concentration in the plasma and extracellular fluid. Most of its multiple actions contribute to this effect and the rate of secretion of the parathyroid glands is inversely related to the circulating calcium concentration, providing close negative feedback control of the plasma calcium level. PTH accelerates bone breakdown and increases renal calcium retention and intestinal calcium absorption, this being the order of relative contribution of these actions to the hypercalcaemia elicited by injecting a large dose. Consideration of their individual dose-response curves, however, suggested a very different relative importance in normal physiology and led us to make a direct test of the effects of infusing minute doses continuously for periods of several weeks.

A miniature syringe-type pump (the Mill Hill infuser) was developed for us by the Engineering Division of the National Institute for Medical Research to enable continuous infusion during investigations and experimental therapy in freely mobile patients and large animals². Large dogs, whose calcium metabolism is similar to that of man, were equipped with indwelling cannulae in the external jugular vein³ and trained to wear the infusers on a light leather harness. Two cannulae were used, a technique permitting withdrawal of large well-mixed blood samples without interruption of the hormone infusion.

The results of infusions lasting up to 3 weeks are illustrated in Fig. 1. A dose rate of $25 \text{ ng kg}^{-1} \text{ h}^{-1}$ ($0.05 \text{ U kg}^{-1} \text{ h}^{-1}$) continued for 3 d left the plasma calcium level of two dogs unchanged, but $100 \text{ ng kg}^{-1} \text{ h}^{-1}$ caused marked hypercalcaemia in each of four animals, reaching a plateau within 24 h and continuing throughout the infusions.

These results permit conclusions that could not be drawn from experiments by subcutaneous injection, in which the rate of absorption and the extent of local destruction of the hormone are both uncontrolled. For example, they make possible an estimate of the plasma level of intact PTH required to produce marked hypercalcaemia. From published estimates of the half time of disappearance of intact PTH (approximately 0.1 h (refs 8–10)), it can be calculated that an infusion of $0.1 \mu\text{g kg}^{-1} \text{ h}^{-1}$ could only support an equilibrium circulating level of about 30 pg ml^{-1} of intact hormone. From the 'black box' relationship: content at equilibrium = (entry rate $\times t_{1/2}$)/0.693. Content is converted to concentration by assuming distribution throughout body water (50% of body weight). This estimate of the hypercalcaemic level is strikingly lower than present radioimmunoassay estimates of the PTH level in normal health (for example 1 ng ml^{-1} (ref. 11)). The discrepancy underlines the fact that immunoassays can estimate biological activity only very indirectly. For example, biological activity of PTH is associated with N-terminal fragments, whereas current immunoassays for this hormone measure principally C-terminal fragments of long half life^{1,12,13}.

The results in Fig. 1 also give an indication of the normal rate of parathyroid secretion. The hypercalcaemia shows that the circulating level of bioactive PTH must have been greatly increased by infusion of $100 \text{ ng kg}^{-1} \text{ h}^{-1}$, although on immunoassay evidence the output of the animal's own parathyroids was presumably reduced virtually to zero at the calcium concentration reached^{1,14}. Copp and his colleagues¹⁵, using crude parathyroid extract, estimated that the dose required to maintain plasma calcium in acutely parathyroidectomised dogs kept under anaesthesia was $0.1 \text{ U kg}^{-1} \text{ h}^{-1}$. This estimate of the endogenous secretion rate is close to that which the present evidence suggests, in spite of the many differences in method.

At first sight the evidence obtained on the mechanisms contributing to hypercalcaemia at this low level is surprising. For example, signs of osteolysis were looked for in bone biopsy samples from the iliac crest before and after each infusion and alcohol-soluble hydroxyproline was measured in the plasma (an index of collagen breakdown¹⁶ which correlates closely with bone destruction^{17,18}). The biopsies, examined by Darby⁴, showed no increase in osteoclast numbers or in the areas involved in resorption and the hydroxyproline levels did not increase during hypercalcaemia in any of the four animals (Table 1).

Little support, however, can be found for the assumption that the acceleration of bone breakdown is a normal physiological response to parathyroid hormone. It occurs in patients with an actively secreting parathyroid adenoma and is known to be a direct effect of exposing bone to high concentrations of PTH (ref. 1). But the doses required to elicit a half-maximal hypercalcaemic response involving calcium mobilisation from bone are of the order of

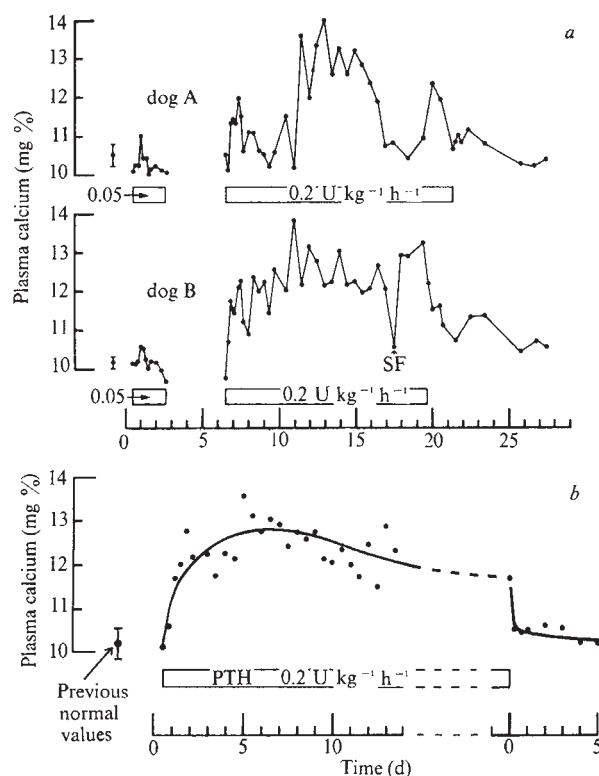


Fig. 1 *a*, Plasma calcium levels of two dogs during and after infusions of highly purified bovine parathyroid hormone at 25 and $100 \text{ ng kg}^{-1} \text{ h}^{-1}$ (0.05 and $0.2 \text{ MRC units kg}^{-1} \text{ h}^{-1}$). Horizontal bars indicate the duration of the infusions, which were separated by an interval of 3 d. The mean and s.d. of the plasma calcium level during 5 d preceding the first infusion is shown on the left. The letters SF denote a temporary syringe failure. Preliminary descriptions of these experiments were presented at the Endocrinology 1973 meeting⁴ and the Tenth European Symposium on Calcified Tissues⁵. Infusions were carried out at 2 ml per 24 h, in a vehicle consisting of 10% dog serum in 1% (w/v) sodium acetate trihydrate. This was heated at 56°C for 1 h ($\text{pH} \approx 7$) to destroy proteolytic enzymes, adjusted to pH 4 with concentrated HCl, sterilised by membrane filtration (Millipore membranes, mean pore size $0.45 \mu\text{m}$) and stored at -20°C . It is a nonantigenic variant of the vehicle used in our laboratory for bioassay of parathyroid hormone⁶. PTH dissolved in this vehicle at only a few $\mu\text{g ml}^{-1}$ was shown by the intravenous chick assay to be protected from surface absorption and to be stable for at least one week at room temperature. Calcium and phosphate analyses were carried out by atomic absorption spectrophotometry⁷ and had a coefficient of variation of less than 1%. *b*, Mean values of the plasma calcium level at corresponding times during infusions at the hypercalcaemic rate ($100 \text{ ng kg}^{-1} \text{ h}^{-1}$) to four dogs. As two infusions lasted for 2 weeks and two for 3 weeks, the time scale is interrupted and restarts at the end of the infusions. The solid line is drawn to give an overall impression of the average effect, emphasising the slow onset of hypercalcaemia and the rapid fall in calcium level which followed termination of the infusion in all dogs. This time course is consistent with the known slowness of induction of enhanced intestinal absorption and rapid recovery from the renal action¹. PTH, parathyroid hormone.

20 U kg^{-1} in dogs³ and 50 U kg^{-1} in chicks⁶—both sensitive species. Experiments including potentiation of the PTH response with a small intravenous calcium injection suggest that the slow osteolytic process is initiated by (and long outlasts) a very rapid cellular response involving calcium influx¹ as well as activation of adenylyl cyclase¹⁹, but these responses required doses of hormone corresponding to initial plasma levels thousands of times greater than the hypercalcaemic level calculated above for the present infusion study.

The hypercalcaemia due to infusion of $100 \text{ ng kg}^{-1} \text{ h}^{-1}$ therefore presumably involves more sensitive responses and we looked for effects on calcium absorp-

tion and excretion. The calcium absorption coefficient (α =calcium absorbed/calcium ingested) was measured by giving ^{47}Ca by mouth and ^{45}Ca intravenously²⁰ and following the isotope ratio in plasma until it became constant. Analysis of urine from these large dogs was achieved only by housing two of them in metabolic cages for 24 h and comparing the concentrations of calcium and creatinine.

A striking effect on calcium absorption was observed during each infusion, α rising to between two and four times control values⁴. This action of PTH is well established¹, but its relative sensitivity was previously unknown. Its mechanism may involve enhanced production of 1,25 dihydroxycholecalciferol, a biologically active metabolite of vitamin D (refs 21 and 22) and induction of the vitamin D-dependent calcium-binding protein described by Wasserman and Taylor²³.

The urinary calcium: creatinine ratio fell during PTH infusion in both dogs studied, in spite of the marked hypercalcaemia and consequent increase in filtered load. This calcium-retaining action of the hormone is also well established¹ and was recently studied in dogs by micropuncture during infusion of $2 \text{ U kg}^{-1} \text{ h}^{-1}$, a dose which greatly enhanced distal tubular reabsorption²⁴. Interestingly, the phosphaturic action of PTH has a proximal tubular mechanism²⁵, so presumably there are two anatomically separate sets of PTH receptors in the kidney alone. Although phosphaturia is known to be evoked by lower doses than bone breakdown²⁶, neither urine nor plasma phosphate concentrations changed during the present infusions.

Table 1 Plasma hydroxyproline ($\mu\text{g ml}^{-1}$)

Dog	Control	During hypercalcaemia
A	7.2	7.2
B	4.2	3.0
C	3.5	3.2
D	4.5	4.0

Heparinised plasma for analysis (1.5 ml) was obtained by pooling aliquots from 3–5 successive days during control or infusion periods, each sample having been separated within 30 min and stored at -20°C . Non-protein bound (alcohol-soluble) hydroxyproline was measured by the method of Le Roy, *et al.*¹⁶.

These findings suggest the need to reassess current views of the biological role and clinical significance of parathyroid hormone. The contrast between the effects of administration in a physiological manner and the well known response to single or intermittent injections is so great as to virtually reverse the currently accepted picture of parathyroid hormone as an agent of bone destruction. The evidence indicates rather that its physiological role may be to promote calcium retention and bone formation and focuses attention on earlier studies in which PTH given chronically to rats in low dose was shown to have an anabolic effect on bone^{27–29}.

This conclusion has clinical implications of two kinds. Pathologically, the qualitative difference between high-dose and low-doses responses to parathyroid hormone implies that the syndrome of hyperparathyroidism may need to be subdivided. The demineralising condition currently regarded as typical may result only when there is gross over-secretion by the gland³⁰, though it will be difficult to prove this until radioimmunoassay findings can be translated into circulating levels of bioactive PTH. Manifestations of milder forms of the disease, including the frequent formation of renal calculi, are probably much influenced by the dietary levels of calcium and vitamin D (ref. 31), but it is particularly interesting that there is evidence for general or localised increase in bone density in some cases of

primary³² and secondary³³ hyperparathyroidism. Therapeutically, the present study strengthens the case for clinical trial of parathyroid hormone in osteoporosis, alone or in combination with calcitonin³⁴. As it seems that the renal and intestinal effects of parathyroid hormone can be obtained at a dose level which does not significantly increase bone breakdown, low doses may be capable of inducing a positive calcium balance and enhancing bone formation.

Finally, the importance of close attention to physiological dose levels and entry rates in future studies of hormone action can be briefly illustrated from other recent findings. The very name 'vasopressin', commonly used as a synonym for antidiuretic hormone, provides an example of a misleading physiological conclusion reached on the basis of inappropriate dosage. The word suggests a vasoconstrictor and pressor agent, yet pressor effects are first seen after doses of $10\text{--}40 \mu\text{g}$ ($5\text{--}20 \text{ U}$) in man³⁵, which must produce blood levels of several ng ml^{-1} . The normal blood level of this hormone is known now to be about 4 pg ml^{-1} ($2 \mu\text{U ml}^{-1}$), rising to 40 pg ml^{-1} during water deprivation³⁶. At this concentration, the hormone seems to act exclusively on the kidney. The need to imitate physiological entry rates was also illustrated by work of Knobil and his colleagues^{37,38}, showing that the 'pre-ovulatory' surge of luteinising hormone can be triggered by a small dose of oestrogen in ovariectomised monkeys. There was no dose which would produce the response unless a physiological basal oestrogen level had been established during the previous week by subcutaneous injections of oestrogen in oil or implanting crystals in a slow-release capsule.

Insulin is another hormone with a wide variety of dose-related effects and it may be that the incidence of pathological changes in chronically maintained diabetics could be greatly reduced by imitating physiological blood levels more closely than the depot-insulins permit.

Parathyroid hormone was prepared from fresh-frozen beef glands in the large scale laboratory of the National Institute for Medical Research. We thank Dr J. L. H. O'Riordan, Middlesex Hospital, for assistance in the final stages of its purification and the Armour Pharmaceutical Company, Eastbourne, who provided facilities for sterile ampouling.

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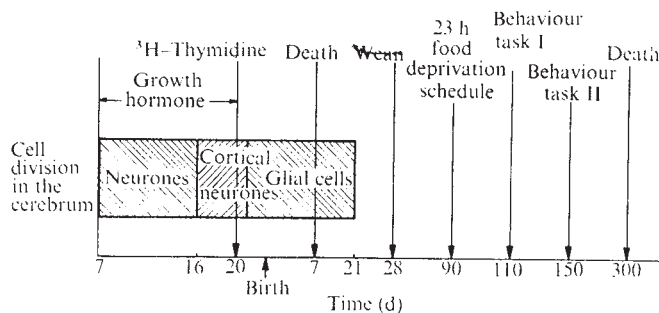


Fig. 1 Time course for the experimental procedure.

received the same volume of vehicle alone. Injections were given from day 7 of gestation, at the time of formation of the neural tube, until day 20 when each pregnant rat was injected intraperitoneally with 500 μ Ci of tritiated thymidine (3 H-TdR). Neurones in the cerebral cortex were labelled specifically by administration of 3 H-TdR at the time of their origin on day 20 of gestation². The rate of

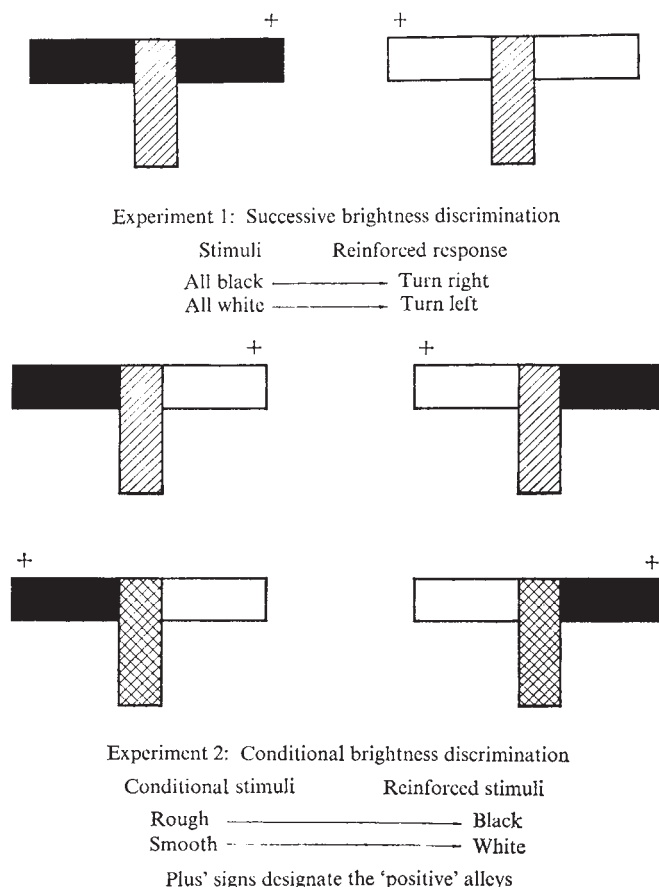


Fig. 2 Procedures used in the conditional discrimination tasks. Experiment 1: successive brightness or spatial conditional discrimination where the animal is trained to turn right for reinforcement when both arms are black, and left when both are white. Experiment 2: brightness conditional discrimination where the animal is trained to turn to the black arm for reinforcement when the floor of the alley is rough, and to the white arm when the alley floor is smooth. +, Denotes the positively reinforced discriminanda and the cross-hatching of the alley arm represents the rough tactile stimulus.

Prenatal action of growth hormone on brain and behaviour

IMPLICIT in all physiological theories of learning is the concept that learning ability depends on neuronal interaction, a function of both the number of neurones and the extent of their interconnections. Although learning ability varies with axo-dendritic development¹, a similar relationship to neuronal number has not been demonstrated, possibly because neuronal proliferation in most species, including rat² and man³, has ceased *in utero*. As alterations in neuronal number must therefore occur before birth we hypothesised that any prenatal growth factor would increase the number of cortical neurones and enhance subsequent learning ability. We have investigated this question, using young rats which had received pituitary growth hormone as the prenatal growth stimulus.

The experimental procedure is summarised in Fig. 1. Pregnant Wistar rats were treated at random in one of five ways: experimental animals received daily subcutaneous injections of either 3 mg, 1 mg, 100 μ g or 10 μ g of purified porcine growth hormone (0.6 U mg⁻¹) and controls

Table 1 Effect of various doses of growth hormone on brain structure and learning ability

Group	Brain weight* (mg)	³ H-TdR uptake* into brain DNA (d.p.m.)	Packing* density (d.p.m. mg ⁻¹ of brain tissue)	Number of trials† to obtain criterion performance on the first behavioural task‡	Number of trials† to obtain criterion performance on the second behavioural task‡
Control	387 ± 81	14,497 ± 4,763	43 ± 15	36 ± 12	88 ± 25
3 mg PGH	566 ± 50	30,703 ± 5,703	54 ± 11	24 ± 7	59 ± 6
1 mg PGH	562 ± 60	23,480 ± 6,458	43 ± 11	21 ± 3	30 ± 27
100 µg PGH	640 ± 55	25,380 ± 7,755	41 ± 12	18 ± 5	30 ± 19
10 µg PGH	451 ± 63	23,069 ± 1,771	53 ± 7	25 ± 7	51 ± 10
F	43.4	8.2	2.3	11.1	14.8
d.f.	4,70	4,43	4,43	4,63	4,62
P	<0.001	<0.001	>0.05	<0.001	<0.001

Results are expressed as mean ± s.d. PGH, porcine growth hormone.

* Data analysed by a one-way fixed effects analysis of variance for unequal group sizes.

† Data analysed by a two-way fixed effects analysis of variance of unweighted means for unequal group sizes.

‡ A learning criterion of 10 consecutive correct responses was used.

proliferation within this discrete population was determined subsequently by the incorporation of ³H-TdR into brain DNA, and the validity of the procedure was assessed by fine-resolution autoradiographic localisation. On the seventh day after birth the brains of half the offspring in each litter were examined. DNA⁴ was extracted from each brain and its radioactivity was determined. Autoradiograms were prepared from one animal in each litter.

The remaining offspring were examined for learning ability at maturity. On day 90 they were placed on a 23 h food deprivation schedule and tested for performance on a series of conditional discrimination tasks of increasing order of difficulty (Fig. 2). Animals were killed on day 300, blood collected and plasma concentrations of thyroxine⁵, testosterone⁶, insulin⁷ and corticosterone⁸ in response to stress measured to determine any hormonal changes which may have influenced behaviour.

Table 1 shows that growth hormone produced a significant increase in brain weight and cellular content. The number of cortical neurones increased as measured by the incorporation of ³H-TdR and subsequent localisation by autoradiography. In all autoradiograms a band of heavily labelled cells was found only in the supragranular layers of the cortex, indicating that ³H-TdR uptake specifically reflects the proliferation of neurones within this region. When examined for functional capacity, offspring of mothers treated with growth hormone during pregnancy showed enhanced ability, obtaining criterion performance in significantly fewer trials than controls. This difference became particularly apparent on the second two-cue learning task, where after 100 free trials, 63% of control animals had failed to reach criterion performance. In comparison, only one animal treated with growth hormone was unsuccessful by this time. Enhanced performance could not be attributed to activation changes as determined by both latency of responding and hormonal response to stress. No change was found in adult body weight or plasma hormone concentrations ($P > 0.05$). Indeed at maturity, brain structure and functional capacity seemed to be the sole consequences of growth hormone treatment.

These results show that the administration of growth hormone during the time of neuronal proliferation produces a permanent increase in the number of cortical neurones and that there is a subsequent enhancement of learning ability. Although this period has been largely neglected as a vulnerable period of brain development, these findings demonstrate that it is critical not only for growth but for subsequent intellectual capacity.

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Depolarisation of *Onchidium* neurone by glycine

INTRACELLULAR recording of cholinergic synapses in the *Onchidium* oesophageal ganglia revealed one neurone, V-3, in the visceral ganglion in which neither membrane potential nor conductance changes after perfusion with 0.5 mM acetylcholine. Significant depolarisation with increased membrane conductance was caused by 1.3 mM glycine. This response to glycine is opposite to that of the mammalian spinal motoneurone¹⁻³. Neurones examined in the ganglia, except V-3, were not affected by glycine. Here we report the ionic mechanism of glycine depolarisation, and compare the effect of strychnine on glycine depolarisation with its antagonistic action of mammalian glycine inhibition⁴.

The excised oesophageal ganglia were continuously perfused with artificial seawater (NaCl 457.6 mM, KCl 9.6 mM, CaCl₂ 10.4 mM, MgCl₂ 48.5 mM, pH, 7.8 by Tris-HCl, at 23° C). Two microelectrodes were inserted into the V-3 neurone, one for recording the membrane potential, the other for applying current to polarise the membrane. The resting membrane potential was -56.3 ± 4.9 mV (mean ± s.d.) (30 cells). Some spontaneous excitatory postsynaptic potentials, but no spontaneous firings were observed. The cells were depolarised about 40 mV with the membrane conductance increased to about twice normal and they fired vigorously when 13 mM glycine was added to the perfusing solution. Ouabain (3×10^{-4} M) had no effect on the level of resting potential or glycine depolarisation. Thus, the depolarisation is probably not caused by the inactivation of an electrogenic Na pump⁵ or the activation of the outward Cl pump⁶. The glycine depolarisation did not change significantly when the chloride ions in the bathing solution were replaced by

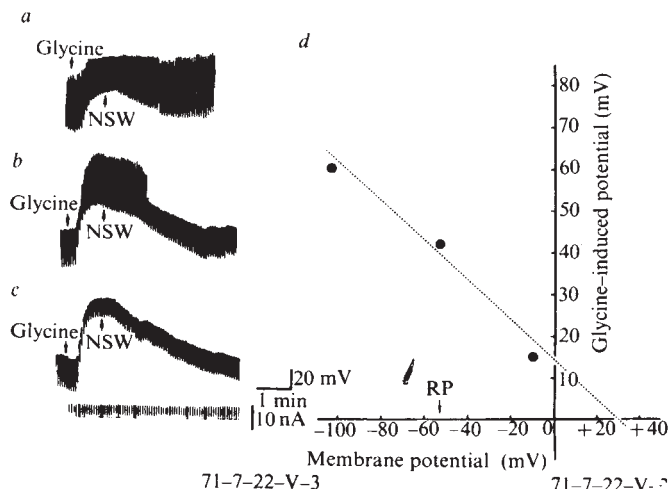


Fig. 1 Effect of displacement of membrane potential on amplitude of glycine depolarisation (glycine, 13 mM). *b*, Glycine depolarisation at original resting potential (-53 mV); *a*, at depolarised membrane potential of 43 mV; *c*, at hyperpolarised membrane potential of 50 mV; *d*, Amplitude of glycine depolarisation plotted against three different levels of membrane potential. The membrane potential at the point where the dotted line crosses the abscissa was taken as the equilibrium potential for glycine depolarisation. RP, original resting membrane potential (-53 mV). NSW, normal seawater.

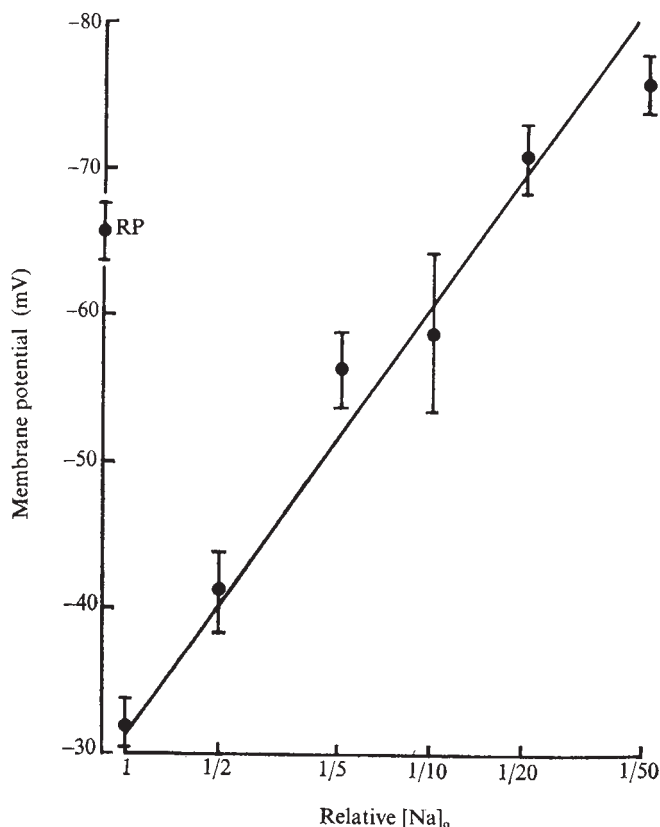


Fig. 2 Relationship between amplitude of glycine (13 mM) depolarisation and $[Na]_o$ in seawater. Amplitude of glycine depolarisation (mean $\pm$ s.d., three V-3 neurones) plotted against $[Na]_o$. Average of original resting membrane potential is shown as RP. The resting potential was stepwise hyperpolarised a total of approximately 17 mV by stepwise decrease from 1 to 1/50 in $[Na]_o$. Thus each glycine depolarisation was the shift from the new resting potential at each new $[Na]_o$ concentration.

acetate. Increase of $[K]_o$ to twice normal did not change glycine depolarisation.

To determine the equilibrium potential for glycine depolarisation, the membrane potential was changed by current application. Figure 1 shows an equilibrium potential of +30 mV obtained by extrapolation of three experimental values. This high potential indicates that the ions involved in the generation of glycine depolarisation are probably Na, or Ca ions, or both.

The next series of experiments therefore consisted in changing $[Na]_o$ to modify this concentration gradient across the cell membrane (NaCl replaced by Tris-Cl). Stepwise decrease in $[Na]_o$ from 457.6 to 9.1 mM caused a corresponding stepwise decrease in the level of depolarisation by 13 mM glycine (Fig. 2). Note that the normal resting potential was hyperpolarised in steps corresponding to the stepwise decrease in $[Na]_o$ from 1 to 1/50. The total amount of hyperpolarisation was approximately 17 mV. The reported depolarisation values are those measured from each respective new 'resting potential'. The change in depolarisation for 10 times change in $[Na]_o$ was about 30 mV. Tetrodotoxin (3×10^{-5} M) had no effect on glycine depolarisation. This indicates that the glycine effect is probably taking place at the subsynaptic membrane⁷⁻⁹. For a five-fold increase of $[Ca]_o$, the glycine depolarisation remained at the same level. Hence, it may be concluded that glycine depolarisation is due exclusively to an increased permeability of the membrane to Na. Doubling the concentration of Mg (97 mM) in seawater had no effect on the glycine depolarisation. The lack of effect of Mg as well as Ca indicates that the glycine action is subsynaptic rather than presynaptic. Glycine was also effective when applied locally to the soma and neck of the V-3 neurone by passive diffusion from a glass microelectrode (tip diameter, about 3 μ m). The neck portion of this neurone was much more sensitive to glycine than was the soma. Synapses of the *Onchidium* nervous system are always axo-axonic and are located in the neuropile at various distances from the soma (T. Yamamoto, unpublished observation).

Interestingly, the effect of glycine on the V-3 neurone was augmented, not blocked, by 2.5×10^{-4} M strychnine. Figure 3a shows depolarisations at various concentrations of glycine from 0.1 to 130 mM; Fig. 3b shows the augmenting effect of strychnine on each glycine depolarisation. The membrane conductance at the plateau phase of depolarisation by glycine plus strychnine (Fig. 3b, right) was certainly no larger than that from glycine alone (Fig. 3a right). It may even have been smaller. Strychnine alone in this concentration had no detectable effect on the resting

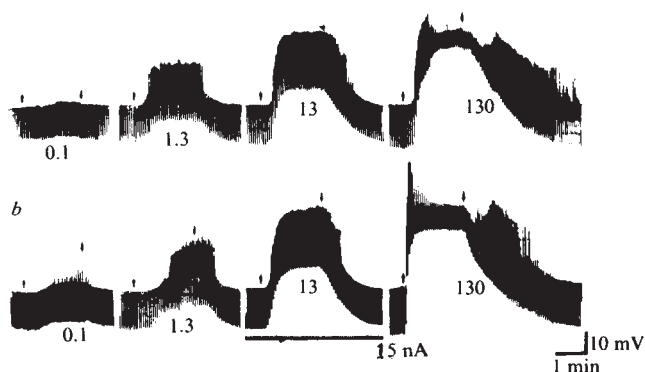


Fig. 3 Augmenting effect of strychnine on glycine depolarisation at various concentrations of glycine. *a*, Glycine alone; *b*, glycine (0.1 to 130 mM) plus 2.5×10^{-4} M strychnine. Figures indicate glycine concentration (mM). Strychnine alone had no effect on the resting membrane potential or conductance.

membrane property, in contrast to its effect on the giant axon of the lobster⁹. The equilibrium potential of glycine depolarisation was 22.7 ± 2.7 mV (four cells); that of glycine plus strychnine was 41.4 ± 3.2 mV. The increase in membrane conductance was $58.3 \pm 16.2\%$ in the former and $44.7 \pm 5.1\%$ in the latter. The differences in equilibrium potential and membrane conductance change for glycine plus strychnine compared with glycine alone indicate that the strychnine augmentation is caused by a decrease in K permeability.

Mention of the effect of GABA on the V-3 neurone may be worthwhile. α -Amino butyric acid (GABA, 10 mM) caused a few mV depolarisations of the V-3 neurone. At the same time it caused a decrease in membrane conductance to about 80% of normal. Preliminary experiments indicate that this depolarisation results in a decrease of the resting K permeability of the subsynaptic membrane. This effect is completely different from that on the vertebrate pre and postsynaptic membranes. Both membranes are depolarised by GABA due to increased Na and Cl permeability respectively¹⁰⁻¹².

In summary, glycine is excitatory in the V-3 neurone. Glycine depolarisation is due solely to increase in Na permeability at the subsynaptic membrane. Strychnine augmentation is caused by K permeability decrease during the glycine depolarisation. The effects of glycine and GABA on the membrane properties of *Onchidium* are very different from their effects on vertebrate neurones.

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Leptospiral motility

THE motility of spirochaetes, especially the *Leptospira*, has interested many workers but has remained unexplained despite the application of modern techniques. Inadequate observations by Noguchi¹ have been used by modern workers (Ritchie and Ellinghausen²; Nauman³) to construct inaccurate models of leptospiral movement, the main inaccuracy of which is that leptospirae rotate upon their axes in free liquid. Noguchi¹, however, did observe that "when

one end is straight and the other semicircularly hooked, the organism usually progresses in the direction of the straight portion and seems to be propelled from the rear by the rotating hook". Although not correct in important details, the observation of translational movement in the direction of a straightened portion is a fundamental principle. Previous observations and explanations were at variance with the physical principles of fluid mechanics. This paper presents information which we believe to be in accord with such principles.

Observations were made using a Reichert Zetopan microscope set for dark ground operation. Videorecordings were made to aid the categorising of the movements, and direct observation was used to analyse the finer details. The organism used was *Leptospira icterohaemorrhagiae* strain 3H cultured in Korthof medium. Particulate matter was supplied to the organism either as particles of agar added to the culture on the microscope slide, or by allowing an active culture to invade semi-solid nutrient agar.

Movement in free liquid. With non-translational movement (Fig. 1a) the body remains relatively stationary with

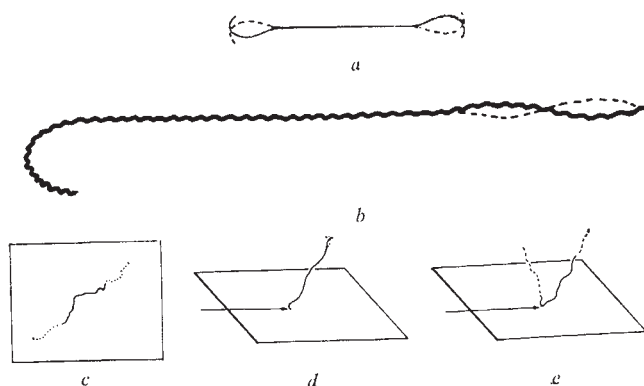


Fig. 1 Motility of *Leptospira icterohaemorrhagiae*. a, Non-translational movement in free liquid; b, translational movement in free liquid; c, crawling on a smooth surface; d, anchoring on a smooth surface; e, staple movement on a smooth surface. Arrows indicate point of fixation.

either end waving in a circular motion in opposite directions. The rapid movement gives the appearance of spinning and the hooked ends blur into the familiar 'button hole' figure. Illumination causes the organisms to move more slowly and under such conditions contra-rotation of the hooked ends may be observed. Though care must be exercised in extrapolating from the behaviour of such organisms to the normal state, the observation that organisms decelerate smoothly would suggest that an abrupt change is unlikely. The forces produced by this type of movement are seen to be equal in all directions as there is no net change in the position of the organism.

When movement is Translational (Fig. 1b), the end towards which the translation is effected performs helical waves travelling for a short distance of the length of the cell towards the trailing end. The trailing end forms a broad hook which waves in an irregular circle in the opposite direction to the propulsive helical wave to provide stability and prevent rotation of the whole body. The helical wave may also be observed when a portion of the body is fixed to or embedded in a particle and unable to rotate. An organism has been observed moving with a piece of colloidal graphite fixed to the tip of the trailing end. The particle was so massive with respect to the organism that there was no tendency for the body to rotate, emphasising the function of the hooked tail.

Movement on a smooth substrate. *Leptospira* may crawl (Fig. 1c) either like a snake or, alternatively, by lifting clear and then reapplying parts of the body. A variant on this is circular crawling in which no sideways waves of propulsion are seen. The body in this case follows a circular path, the circumference of which is normally less than the length of the body.

The organism may be anchored (Fig. 1d). One end may remain fixed to the substrate despite high rates of flow in the medium. Frequently one end of the organism is seen to be fixed to itself further along, in place of the substrate.

A third possibility is *staple movement* (Fig. 1e) so called because it can be imagined that the organism is passing through a staple or hoop fixed to the substrate. This movement often follows anchoring and is most probably due to the same process. By virtue of the small radius of the protoplasmic cylinder the surface energy would be relatively high, providing the physical adhesion necessary for this type of behaviour. When the organism moves through the 'staple' the spiral of the protoplasmic cylinder behaves as if it were a screw, threading through a small hole. This keeps the cell in intimate contact with the substrate in accordance with the surface energy concept. There is at present no direct evidence to indicate that surface energy forces are responsible for the phenomena observed. Equally, there is no evidence to indicate the presence of adhesive organelles or the secretion of adhesive substances.

Movements in semi-solid medium. Translation is effected by snaking through the medium with the body conforming to the texture of the medium. The body is very flexible. It is not obvious how movement is actually carried out but the body once again screws through the medium.

The most important point to arise from these observations is the notion that when moving in free liquid *Leptospira* do not rotate about the axis as has previously been supposed. The hook at the trailing end of an organism performing translational movement remains relatively still thereby providing an 'inert head' fundamental to the effectiveness of the helical waves of the leading end of the cell as demanded by Taylor's calculations on propulsion by helical waves¹. This basic requirement is not included in previous observations.

A certain amount of roll about the long axis is inevitable as a consequence of the helical nature of the propulsive waves², but this can be seen to be corrected for by flicking movements of the trailing hook. Any remaining rotation could not be confused with the rapid spinning described previously¹⁻³.

The rapid spinning seen in nontranslatory movement is now seen to be an illusion. There is no process available to produce rapid spinning in the absence of flagellae, cilia or jet devices, none of which have been observed. It is, however, a physical reality that the ends can wave in circles of opposite direction, each providing the necessary reaction to the other.

Any attempts to explain these movements at the sub-cellular level must be speculative at the moment. It is possible that the axial filaments might be responsible, provided that they extend along the axis of the body as a whole and are not themselves spiralled structures. This condition seems to be fulfilled³, a fact which eases the problem somewhat, since a system whereby a spiralled filament produces secondary spiral waves defies imagination. The axial filaments are known to extend from each end of the cell for a short distance towards the middle, and it is the terminal portion of the cell that performs the locomotory function.

The importance of these observations to the study of leptospires as pathogens lies in the insight gained as to how the pathogen solves some of the problems posed by both the external environment and the internal environ-

ment of the body of the host.

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Bioassay of a *Drosophila* pheromone influencing sexual selection

PHEROMONES function by transmitting information from a sending to a receiving organism by chemical means. In a classic paper, Bossert and Wilson classified the effects of such information transfer into releasing and priming¹. Releaser pheromones evoke an immediate overt behavioural effect, whereas primer pheromones activate the chemoreceptor in such a way as to alter the physiology of the receiving organism^{1,2}. In general, studies involving the isolation identification of pheromones have concentrated on releasers such as sex attractants, alarm pheromones, trail pheromones and aggregation pheromones³. The advantage of using releaser pheromones in such studies is that the behavioural effects can be observed even in a single individual of the appropriate age, sex and species. This makes the development of a relatively simple and rapid bioassay a straightforward process. So far the important exception to the use of releaser pheromones has been the work of Butler *et al.* showing that 9-ketodecenoic acid inhibits ovarian development in worker bees⁴. This substance, however, was first identified as the sex attractant of the queen bee and later shown to have a primer effect. We describe here a bioassay for a substance with an effect which does not fit easily into either the releaser or primer category.

We have been attempting to isolate and identify the pheromones responsible for the rare male behaviour found in *Drosophila* of several species and in other organisms⁵⁻⁷. Rare male behaviour is characterised by females preferring the strain of males in least abundance presented to them as potential mates. This rare male advantage may have evolutionary significance, since it provides a mechanism for preserving a heterogeneous gene pool.

In particular we are concerned with two intensively studied strains of *Drosophila pseudoobscura*, homozygous Chiricahua (CH) and homozygous Arrowhead (AR) — differing in third chromosome inversions⁸. Previous work has shown that when the ratio of males (CH:AR) is sufficiently different from 1.0, males of the strain in least abundance are favoured as mates by females of both strains (Fig. 1)^{6,7}. Furthermore, a compound or compounds of low polarity extractable from CH males into light petroleum ether (and to a much lesser extent into acetone) has been

shown to be capable of producing an advantage for AR males when the AR:CH ratio is 1.0 (ref. 7).

Attempting to isolate and identify this active material, we have been led to quantify the assay, and in doing so have been able to show that the behaviour can be controlled substantially by olfactory cues alone. In the mating trials, any extract was spotted on to the filter paper bottom of an Elens-Wattiaux observation chamber⁸ and the solvent allowed to evaporate. Twelve aged females from each of the two strains were then introduced unanaesthetised and allowed to acclimate to the chamber for 1 min. Eighteen AR males and six CH males were then introduced in a similar manner and all matings were scored by direct observation with a hand lens. As Fig. 1 shows, this CH:AR ratio of 0.33 normally produced nonrandom mating frequencies favouring the rare CH males; this held true both in clean chambers and in the presence of inactive compounds, solvent or low concentrations of active material. In the presence of petroleum ether extracts of CH males, however (prepared by tissue grinding flies in acetone, centrifuging, decanting and extracting the pellet with petroleum ether, 30°–60° C), this rare male advantage could be removed. As Fig. 2 shows, this removal exhibited a threshold above which random mating (as measured by χ^2) is observed. The relatively high value of this threshold (ten to fifteen flies extracted per fly present) may indicate that the extraction was incomplete or resulted in degradation or loss of the material, that the actual pheromone release was directional (and hence more sparing of material), and/or that other cues (such as auditory, visual or tactile sensations^{9–11}) supplement the olfactory signals. The persistence of random matings even to high multiples of the threshold value lends strength to the supposition that other cues are also important.

This bioassay, and hence the behaviour which it measures, differs significantly from procedures generally used. Unlike the usual assays of releaser pheromones, our bioassay cannot be conducted with individual organisms. The pheromone appears to bias the female's response in a predictable manner, but in a way which cannot be measured for individual females. Furthermore, unlike the situation with primer pheromones, the effect is observable on a very short time scale. In a different context from the reports

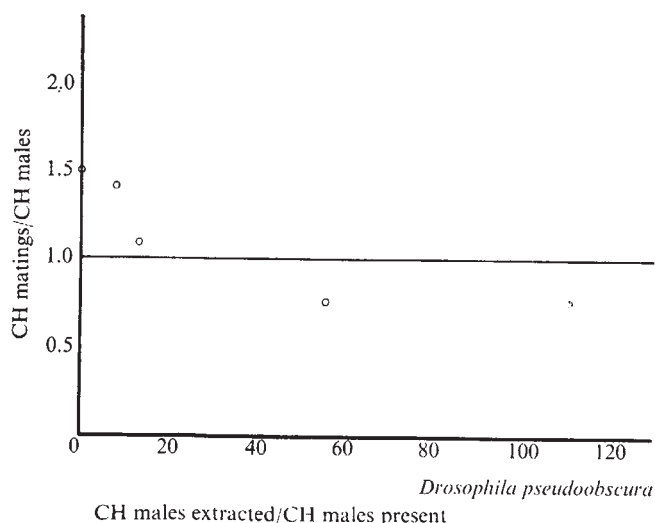


Fig. 1 As the AR:CH ratio approached 50:50, the mating advantage of the CH males disappeared. Ratios are based on a total of 96 observed matings. To guarantee the vitality of the stocks, only chambers in which all females mated are included.

mentioned above, E. O. Wilson has spoken of the "intractable" recognition substances which are "deserving targets for the best efforts of chemists in the future"¹². The females of the *D. pseudoobscura* strains studied are clearly using a chemical cue to determine the presence of males of the CH strain, and altering their behaviour accordingly. Rather than releasing an overt behavioural effect or altering the female's physiology in a long term manner, the

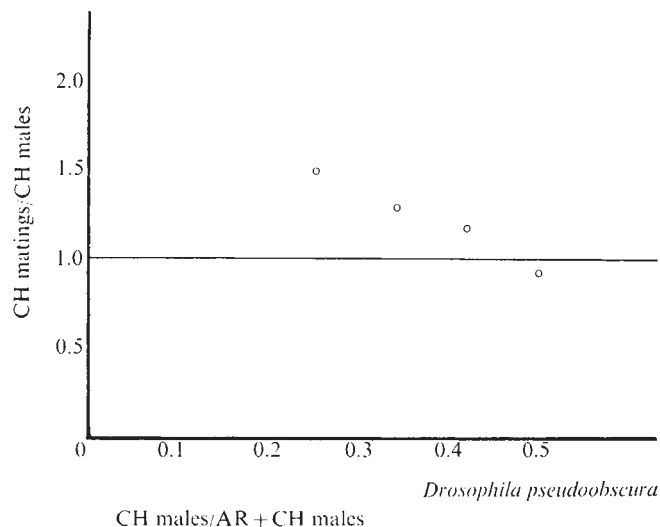


Fig. 2 As further petroleum ether extract of CH males were added to the chambers, the mating advantage of CH males disappeared. Ratios are based on 48 observed matings in chambers where all females mated. The assay can also be run using a 50:50 AR:CH ratio; a full advantage for AR (as determined by the χ^2 for randomness) can be achieved. This assay is used because it appears to provide a better internal control for the vitality of the critical CH flies. There is no significant difference in the statistical stringency of the two assays.

olfactory cue seems to act as information, pure and simple. Although other information (auditory, visual, tactile) is also utilised, the olfactory cue can strongly bias whatever mechanism operates in the females to determine their choice of mates. This work was supported by a US Public Health Service National Institutes of Health research grant.

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Dietary preference and diseases of age

THE frequency of a number of tumour types and several age-related diseases are readily modified by dietary means¹⁻⁸. Traditionally, in defining the influence of a dietary factor experimentally, diets of fixed composition are offered either *ad libitum* or in limited amounts for arbitrarily assigned periods. In *ad libitum* conditions, however, except for the amount of food consumed, the animals have no choice but to eat the diet provided; in restricted feeding conditions, even this option is denied them.

The element of dietary preference was introduced in the investigations reported here. This new approach to long term studies was suggested by the well-established fact that the young rat, unless it has been biased by previous experience or training, has the ability to select for itself essential dietary substances in amounts necessary to sustain life, to grow and to reproduce⁹⁻¹². That this behaviour represents a response to specific metabolic 'needs' is supported by the observations that a variety of conditions which alter metabolism generally results in compensatory changes in appetite for specific food substances¹³⁻²².

The self-selection (S-S) method of feeding seems to have several advantages over a 'fixed' dietary regimen: the diet selected is of physiological significance to the individual, at least during the growth period of life; it more closely approximates the natural condition; and the arbitrary decisions made by the investigator as to the quality of the diet without considering the needs of the individual are avoided. In addition, by permitting the animals to display their preferences, it may be possible to assess on an individual basis the long term consequences of dietary habits at different stages of life.

In this study we provided each rat fed on the S-S basis, three complete isocaloric diets⁸ in separate containers instead of the individual components. They differed only in their casein and sucrose content. To determine the influence of each of the diets separately, other rats were fed *ad libitum* in the conventional manner.

The regimen of the individually housed male Charles River COBS rats, which genetically are relatively heterogeneous, was begun when they were 21 d of age and was maintained for the remainder of the animals' lives (the age at death of the longest lived rat was 1,075 d). The amount of each of the diets consumed by each S-S rat was determined daily. The dietary history during the gestation and suckling periods has been described^{9,23}. Upon the death of each rat, a thorough necropsy was performed and samples of all normal and diseased tissues were taken for histopathological examination. The tissue sections were coded so as to preclude identification with the age and dietary history of the animal.

Far more 'spontaneous' tumours and more cases of kidney, heart, and prostate gland disease occurred among the S-S rats than among the rats fed the same diets singly (Table 1). Indeed, there were a number of tumours for which the incidence among the S-S rats was as great or greater than among all three conventionally fed groups combined. These included benign tumours without respect to type, adenomas and carcinomas without respect to site, leukaemia, tumours of the anterior pituitary, adrenal, exocrine pancreas, and skin. This also held for advanced cases of glomerulonephrosis, myocardial fibrosis, and for prostatitis. Adjustments for time of occurrence, age-specific morbidity rates, population size and length of life, confirmed the differences in risk (Table 1). In addition to the significantly higher incidences, more S-S rats had multiple affections at time of death than did those on the fixed diets; 66% of the S-S rats had at least three of these diseases as compared with 9, 26, and 28% for rats fed low, intermediate, and high protein diets (D₁₀, D₂₂ and D₅₁), respectively.

The higher incidences of the S-S rats were not attributable to longer life spans. Although the S-S rats had a higher life expectancy than rats on the fixed diets, this was limited to age periods before 400 d. Once the various diseases manifested themselves, the life expectancy of the S-S rats was lower than

those of the other groups. The change to higher age-specific mortality rates was commensurate with the consistently higher age-specific morbidity rates.

With the conventional feeding method, the frequencies of the non-neoplastic diseases (Table 1), and of several tumour types⁸, were dependent upon the proportion of protein in the diet.

Table 1 Frequencies of age-related diseases of rats as influenced by mode of feeding

	Dietary regimen*			Self-selection† (D ₁₀ , D ₂₂ , D ₅₁)
	Single-fixed D ₁₀	Single-fixed D ₂₂	Single-fixed D ₅₁	
	Cases per 100 rats‡			
Tumour bearing rats	26	29	28	62'' (177)††
Tumours, all types	29	38	39	91'' (295)
Benign	20	28	30	7'' (232)
Malignant	9	10	9	14 (107)
Glomerulonephrosis				
All grades	38	56	73§	90** (145)
Moderate and severe	4	18	39§	56** (176)
Myocardial fibrosis				
All grades	11	42	48§	67** (167)
Moderate and severe	<1	4	18§	38** (258)
Prostatitis	5	10	12¶	62'' (639)

*Diets identified according to the proportion of the casein component.

†Average casein content of the composite diet selected throughout the greater part of life, between 27-28%.

‡Number of rats: 125 in the S-S group, 250 in each group provided one diet only.

§ $P < 10^{-3}$, ¶ $P < 0.01$; disease frequency for D₅₁ significantly different from D₁₀. Absence of superscript indicates difference between groups, N. S. Fisher's exact probability test applied to empirical data. χ^2 test of association among the 3 groups significant for all grades and for moderate and severe cases of glomerulonephrosis and myocardial fibrosis ($P < 10^{-3}$; $< 10^{-6}$; $< 10^{-6}$; $< 10^{-4}$, respectively).

** $P < 10^{-8}$; ** $P < 0.003$; S-S frequency significantly higher than that of the fixed dietary group with the highest frequency.

††Relative morbidity ratio ($\times 100$), all ages. Values of 100 assigned to the fixed dietary group with the highest frequency. Values in the S-S group > 100 indicate extent of increase in risk relative to that of the fixed dietary group. Morbidity ratio = $c_x / \bar{c}_x$, where c_x = number of cases observed in the S-S group and $\bar{c}_x$ = expected number of cases: $\bar{c}_x = (\bar{c}_{x1} \times I_{x1}) + (\bar{c}_{x2} \times I_{x2}) + (\bar{c}_{x3} \times I_{x3}) \dots$ where $\bar{c}_{x1,2,3, \dots}$ = 'standard' age-specific morbidity rates, consecutive 100 d periods and $I_{x1,2,3, \dots}$ = exposure (that is, number of living S-S rats entering an age period) consecutive 100 d periods. For each disease class, age-specific morbidity rates of the fixed dietary group with the highest frequency used as standard rates.

The increase in the prevalence of these diseases for the S-S rats cannot be explained on this basis as the protein/carbohydrate ratio of the diet selected by any of the S-S rats had to fall within the upper and lower limits of the three fixed diets.

The S-S rats grew more rapidly and attained higher body weights than rats fed the diets separately. We had shown^{23,24} that among conventionally fed rats those which grew more rapidly and attained higher body weights during maturity had a higher risk of tumours than those with lower rates of growth and lower maximum body weights. Body weight differences, however, do not explain the marked increase in disease proneness of the S-S rats. For this analysis, each of the S-S rats was weight-paired with individuals from the D₂₂ group. There was,

consistently, a greater number of tumour bearing rats in the S-S group than among conventionally fed rats. For example, on the basis of the body weight at 98 d old, 60% of the S-S rats but only 23% of rats of corresponding body weights from the D₂₂ group subsequently developed tumours. When maximum body weights were matched, 58 and 33% of the rats maintained on the S-S and D₂₂ regimens, respectively, developed tumours.

No two S-S rats exhibited the same dietary preferences. After an initial rapid increase in the amount of food consumed, the daily intake by each rat remained relatively uniform from 11 to as much as 49 weeks. By 1 y of age, 90% of the rats showed a

Table 2 Dietary preference and disease frequency

	Dietary preference *†					
	Casein content of diet (%)‡			Food intake (g)§		
	Low	Inter- mediate	High	Low	Inter- mediate	High
	Disease frequency (%)					
Tumour-bearing rats	67	70	54	60	63	68
Benign tumours	75	90	73	75	85	78
Malignant tumours	20	15	10	13	13	20
Epithelial tumours¶, all Adenomas	78	90**	59	70	78	78
Endocrine tumours¶, all Anterior pituitary	58	58	46	48	55	59
Pancreatic	55	58	42	48	53	54
Glomerulonephrosis**	40	33	29	28	35	39
Myocardial fibrosis**	13	13	10	18	10	7
Prostatitis	88	98	95	90	95	95
	60	73	76	65	70	73
	60	58	76	68	60	66

*†Rats classed according to dietary choice during 200–300 d of age.

†Number of rats in each subclass was 40 or 41 (4 rats died before 100 d).

‡Class limits: low = 12.0–24.8%; intermediate = 25.2–30.3%; high = 30.4–43.0%.

§Class limits: low = 16.1–20.2 g; intermediate = 20.3–21.9 g; high = 22.0–26.8 g.

¶Among rats maintained on fixed diets, the highest frequency was: all epithelial tumours, 27%; adenomas, 20%; all endocrine tumours, 20%; anterior pituitary tumours, 10%; pancreatic tumours, 6%. Additional values given in Table 1.

**All grades of severity.

**Frequency of epithelial tumours of the intermediate dietary subclass significantly higher than that of the high-protein subclass ($P < 0.02$). Absence of superscript indicates no significant difference between subclasses. (Fisher's exact probability test applied to empirical data). χ^2 test of association among the three subclasses also not significant.

marked and progressive increase in food intake. Before death only a few animals failed to decrease their intake of food. The level of intake during each of these phases differed widely from rat to rat.

Within the first 5 weeks of feeding, most of the rats established the level of protein and carbohydrate that each would continue to select for a prolonged period. Despite variation in relative preference for the three diets or in the amount of food consumed, the protein: carbohydrate ratio of the resulting composite diet was maintained by some rats for as long as 700 d.

The associations between disease susceptibility and dietary preferences were evaluated by separate rankings of the S-S rats according to the protein content of the composite diet selected and the amount of food consumed. The tumour data are limited to several tumour types, as the numerous tumour classifications that should be made cannot be adequately treated in a short communication.

The data shown in Table 2 for the S-S rats are, in general, representative of the relationships seen at earlier and successive 100 d age periods until 600 d of age was attained. Beyond this time, the progressive decrease in number of animals precluded

further analyses; that is, of the number of rats started in the experiment, 45 and 75% had died by 600 and 700 d, respectively.

The prevalence of the four diseases among the S-S rats, classed according to the protein content of the diet, was almost without exception greater at any level of protein than those of rats maintained on any of the fixed regimens. In addition, whereas the incidences of the renal, myocardial, and prostate gland diseases for the rats on the fixed diets (Table 1) are strongly dependent on the proportion of the protein component of the diet, among the S-S rats the protein dependencies were weak or nonexistent (Table 2). Irrespective of the protein content of the diet the frequency of several types of tumours tended to increase slightly among animals whose food intake was higher. The mean length of life, however, tended to decrease 690 ± 124 , 622 ± 123 , and 580 ± 104 d, respectively). Even so, the differences in the incidences for the neoplastic and non-neoplastic diseases among the subgroups were not significant. In contrast, with fixed regimens the trends for most tumours were consistently striking and statistically significant²⁴.

Unless the members of an experimental population are highly inbred or subjected to extraordinary environmental experiences, not all of the individuals will develop any of the common age-related diseases. In conventional *ad libitum* feeding practices, changes in the prevalence and severity of the observed idiopathic diseases could be taken to represent a dose-response relationship. In S-S conditions, the diet chosen differs from animal to animal and yet equivalent or nearly equivalent effects are exerted by any one diet on disease susceptibility. The dose-response interpretation cannot be applied to the free choice situation. Rather, the impressive increase in the risk of developing tumours or of other diseases of age must be viewed as the direct consequence of the specific selections made by the individual in satisfying its unique metabolic 'needs'.

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Evidence for the inhibition hypothesis in expanded angle illusion

THE illusory expansion of acute angles has long been suspected to be an important contributory factor in a number of well known illusions, such as the Zollner, the Wundt–Hering and the Poggendorf^{1,2}. Recently Blakemore, Carpenter and Georgeson³ proposed that angle expansion is a side effect of an inhibitory process that improves the orientation resolution of the visual system. Neural line and edge detectors considered in isolation, are assumed to respond to a wide range of orientations, but when incorporated into a functional system inhibitory interactions occur between them which sharpen the specification of a line or edge⁴. When two spatially contiguous lines of neighbouring orientations are exposed simultaneously, however, the activity peaks in the population of orientation detectors are shifted away from each other because of the inhibitory interactions. Consequently, the orientations of the lines comprising the angle are perceived wrongly. This process of central lateral inhibition is thought to be similar to that which operates in peripheral sensory structures⁵. It seems natural to ask how far this similarity extends.

Hartline and associates⁶ have described the following major features of inhibition in the compound eye of *Limulus*⁶. First, the distance effect—as the test and inducing stimuli are moved further apart the effectiveness of the inhibition decreases. In the orientation domain experimental evidence indicates that following an initial rapid build up, the illusion diminishes as the angular separation increases^{3,7}. Second, the area effect—the larger the number of neighbouring facets illuminated, the greater the inhibition of the test ommatidium. Extrapolated to the visual cortex, one might expect the induced change in orientation of a test line will be proportional to the number of parallel lines of neighbouring orientation exposed in its vicinity. Experimental data provided by Ogasawara⁸ and Wallace and Crampin⁹ show the larger the number of parallel lines that cross a contour, the greater its angular distortion. If the inducing lines are not parallel then inhibitory interactions will occur between them thus ‘disinhibiting’ their potential combined action on the test line. Evidence that disinhibition does occur in the case of interactions between orientations has been provided by Carpenter and Blakemore⁷. Third, the intensity effect—for a given distance between inducing and test omma-

tidia, the more intense the illumination falling on the inducing facet the more intense the inhibition of the test ommatidium. Where two facets are unequally illuminated, the inhibitory interaction increases the differences in their outputs. Extrapolated to the case of interactions between orientations, these effects suggest the following predictions. If two lines of neighbouring orientations are unequally illuminated, then the angular distortion of the two lines will be unequal because proportionally more inhibition, and therefore more angular distortion, of the dimmer line by the brighter will occur than *vice versa*. Where the luminance of both lines is equal, little change would be expected in the magnitude of the mutual distortion as the absolute luminance is varied over a fairly wide range, as the inhibition should remain proportionately similar. Because verification of these predictions would constitute valuable support for the theory it was decided to test them specifically.

The subject, his chin supported by a rest, looked at the display through a 4 mm artificial pupil with his right eye. The display consisted of a pair of lines, each subtending $2.0^\circ \times 0.17^\circ$, forming an acute angle, whose luminance could be varied independently, or in unison, with neutral density filters. The illusion size was measured in the same way as that employed by Blakemore *et al.*³. A small comparison line, subtending $1.2^\circ \times 0.1^\circ$, was positioned 2.0° from the base line (*B*) of the angle (Fig. 1) by means of a half-silvered mirror. By moving a lever, which rotated a Dove prism, the orientation of the comparison line was adjusted to appear parallel to *B*. The maximum luminance of the angle lines was 8.5 cd m^{-2} and the comparison line was constant at 2.0 cd m^{-2} . Before each estimate of the orientation of *B*, the comparison line was offset $30\text{--}40^\circ$. The subject placed his head on the rest and the room lighting was turned off. The subject adjusted the comparison line, usually in 5–6 s, then the room lighting was turned on and the reading

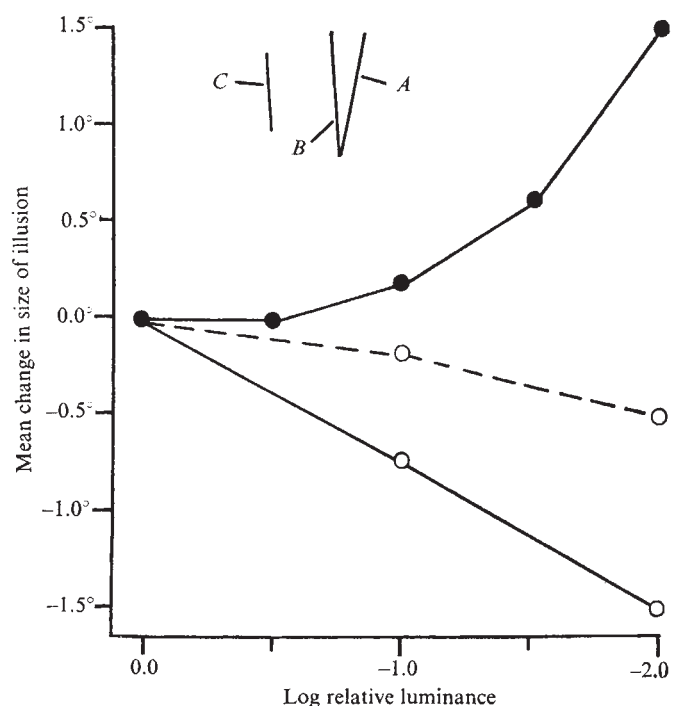


Fig. 1 The inset shows the arrangement of lines used in the present study. *A*, angle line; *B*, base line; *C*, comparison line. Angle of *A* and *B* = 15° . ●—●, *A* constant, luminance of *B* decreased. ○---○, Luminance of *A* and *B* decreased concomitantly. ○—○, *B* constant, luminance of *A* decreased. In all cases illusion magnitude was calculated as orientation of *B* in absence of *A* minus orientation of *B* in presence of *A*.

noted. Each of five subjects made seven estimates of the orientation of *B* under each luminance condition for the three experiments (15 subjects in all). The order in which the estimates were made was changed for each subject.

The results presented in Fig. 1 are for the case where the orientation of *B* with respect to the vertical was 5° anticlockwise, and *A* was positioned 10° clockwise. The upper curve in Fig. 1 shows the progressive increase in the induced tilt of line *B* as it is made dimmer than *A*, indicative of a greater inhibitory effect of *A* on *B*. Analysis of variance on the data showed that this change was significant ($P < 0.01$). The lower curve in Fig. 1 plots the significant reduction ($P < 0.01$) in the induced tilt effect as the luminance of *B* is held constant and that of *A* reduced, indicative of a reduced effect of *A* on *B* as *A* becomes dimmer. The middle curve shows the nonsignificant change in illusion size ($F = 0.735$, d.f. = 2, 8), as the brightness of both *A* and *B* was varied concomitantly over two log units. These results show that the size of the induced tilt effect varies systematically as a function of the relative, but not the absolute luminance of the test and inducing lines. The effect is not confined to this particular orientation of *A* and *B*, nor is it a function of the particular angle used here (15°). The effect is also obtainable when a subjective vertical criterion is used to estimate the orientation of *B*, so the results cannot be due to any interaction with the comparison line.

It is known that the relative luminance of spatially adjacent areas affects their perceived brightness¹⁰. These results show that the relative luminance of orientationally adjacent contours affects their perceived orientation and offer evidence in favour of the hypothesis that angle expansion is the result of an inhibitory process. While the results are similar to a relative contrast phenomenon recently demonstrated for the tilt after effect (TAE)¹¹, the explanation of the present results in terms of adaptation must be rejected for other reasons. The angle expansion illusion is known to be spatial frequency specific¹² while the TAE is not^{11,13}. The TAE also shows an 'indirect effect' when the adapting stimulus is in the region of 90° from the test stimulus¹³, which is not true of the illusion.

The similarities in the operation of inhibition processes in simple visual systems and the interactions between lines indicate that lateral inhibition may be a more general phenomenon than is commonly realised and suggests that certain illusions which involve neighbouring orientations may be explained without the theoretical involvement of constancy mechanisms¹⁴.

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Form-specific colour after effects in scotopic illumination

THE retina of the human eye contains rod and cone receptors, and in very dim light the rods alone function (scotopic illumination) so that objects appear colourless. At higher light levels, cones also function (photopic illumination) and colours are seen.

There is evidence that rods interact only with rods and that the various colour mechanisms, the Stiles π mechanisms, act independently¹⁻³. These events probably occur early in the retina, for the various channels interact even within the retina⁴, and the later stages of the visual system show a strong opponent colour organisation. Other evidence suggests that rod and cone signals interact; in these studies colour sensations are elicited through rods. For example, if one first views a coloured flash that affects cones, a complementary coloured afterimage is seen upon viewing a dim flash that affects only rods⁵. The rods seem to trigger this after image⁶, for its hue remains constant as the wavelength of the dim test flash is varied, and the light level sufficient to trigger the after image coincides with the dark adaptation curve of the rods. Simultaneous contrast colours of blue and green are seen in a bipartite field when one half is illuminated with dim light that stimulates only rods and the other half is illuminated with long wavelength light that stimulates cones⁹⁻¹⁰. Similarly, many colours are seen in a Land two-colour projection produced with two illuminants that stimulate differently long wavelength cones and rods¹¹. These studies suggest that rod and cone signals converge at some level of the visual system.

I have found that form-specific colour after effects—the McCollough effect—can be seen on test patterns observed in dim light that stimulates the rods but not the cones. This shows that rod signals feed into colour mechanisms. McCollough¹² discovered that after adapting for several minutes to a vertical grating in orange light alternating with a horizontal grating in blue light, black and white test gratings with retinal orientation similar to the adapting patterns were tinged with colours complementary to the adapting colours. For example, the vertical test grating appeared blue and the horizontal orange.

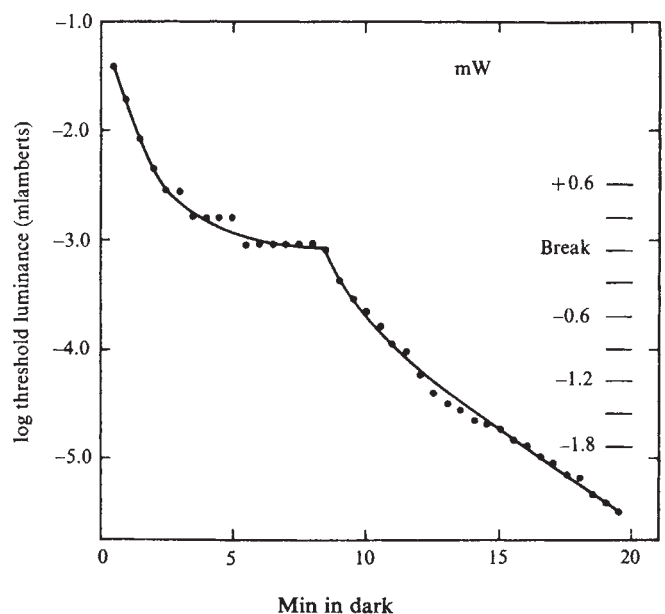


Fig. 1 Dark adaptation curve for one observer. The bars on the right indicate various luminance values for white bars of test patterns.

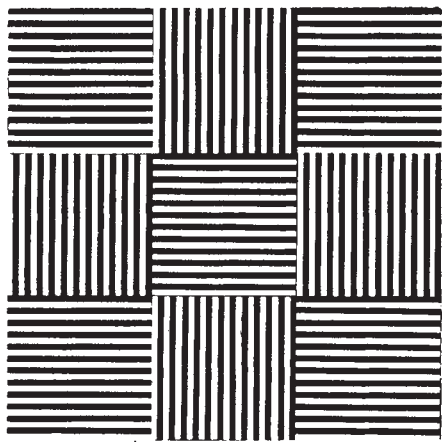


Fig. 2 Test pattern. Gratings had spatial frequency of 0.75 cycles per degree.

Of the three observers I used, two were highly experienced with the after effect and one was uninformed about the experiment. Dark adaptation curves were first measured on the two experienced observers. After strong light adaptation, the observer fixated a tiny red light in the centre of a 10° diameter disk of white paper on a black background and adjusted a neutral wedge, which controlled the illumination level, so that the disk remained just below threshold. These materials had the same reflectance as the test patterns used later. The light came from a regulated projector with accessory lenses and was filtered through a narrow-band yellow-green filter (Wratten 74, dom λ 538nm). The curve for one observer is shown in Fig. 1. The vertical axis represents the luminance of the white disk. The curve for the other observer was very similar. The level of the rod-cone break agrees with the value measured by Chapanis¹³ for a green spot 3° in diameter, 7° from the fovea, which was flashed on for 0.2 s. It was also noticed, subsequently, that the appearance of the test patterns (Figs. 2 and 3) changed markedly near the break: the bars of the gratings appeared sharp just above the break and diffuse just below the break. Rods alone presumably function below the break, for the break is generally assumed to indicate cone threshold.

Colour after effects were next built up with high-contrast square-wave gratings of 0.75 cycles per degree, rear-projected on a diffusing screen. Gratings subtended an area about 30° square. A tiny light was placed in the centre of the screen. Gratings were projected in alternate vertical and horizontal orientations and interchanged every second. On each presentation the gratings were projected in the same position or shifted 180° in phase by mirrors. The shift was determined randomly. The observer stared at the light to help adapt the eyes evenly. The observer adapted for 20 min to the alternating vertical grating in green light (Wratten 40 filter) and the horizontal grating in complementary magenta light (Wratten 31 filter). Mean luminance was about 70 m lamberts. The observer next dark adapted for 30 min and viewed the test pattern (Fig. 2) illuminated with the light used to measure the dark adaptation curves. The luminance of the white areas of the test gratings was set at values indicated by the bars in Fig. 1, starting at the lowest level and moving upward. The spatial frequency of the test pattern was the same as the adapting patterns, 0.75 cycles per degree. The pattern was about 48° square.

The vertical and horizontal gratings of the test pattern appeared faintly pinkish and greenish, respectively, to all observers at the lowest light level, -1.8 log units below the rod-cone break. The colour became more saturated

as the light was increased. The naive observer later matched from memory the strongest scotopic after effects to Munsell colour chips viewed in Macbeth daylight=6750 K. The colours chosen were 10 RP 7/8 and 10 GY 7/8. All observers claimed that the scotopic colours were quite saturated.

The McCollough effect is somewhat specific to the spatial frequency of the adapting pattern¹⁴. The present experiment demonstrates that the colour effect stays tied to the adapting frequency even for test patterns in scotopic light. The previous experiment was essentially repeated using two vertical adapting gratings of 0.5 and 2.0 cycles per degree. The observer again viewed the fixation light in the centre of the adapting field. The patterns were about 30° square and centred on the fixation light. The 0.5 cycles per degree pattern alone was projected in the same position or shifted 180° in phase, as before; the 2.0 cycles per degree pattern was always projected in the same position. Observers adapted for 40–60 min to one grating in green light, alternating with the second grating in magenta light. For some observers, the two gratings were shown for unequal amounts of time to build up good pink and green after-effects. (Low frequency gratings were used, because below the rod-cone break gratings above 5 cycles per degree cannot be resolved¹⁵.) After colour adaptation, the test pattern (Fig. 3) was viewed in Macbeth daylight and then, after dark adaptation, in scotopic light at levels from -1.0 to -0.3 log units below the rod-cone break. The pattern was about 15° wide and 30° high. The test gratings appeared appropriately coloured. For example, adaptation to 0.5 and 2.0 cycles per degree gratings in magenta and green light, respectively, produced a green after effect on the 0.5 cycles per degree test grating and a pink after effect on the 2.0 cycles per degree grating. All observers claimed that the colours were more saturated at scotopic than photopic levels.

That the after effect stays tied to the adapting spatial frequency suggests that the receptive field of the mechanism underlying these effects does not lose its antagonistic surround at scotopic levels. Loss of the surround would cause pattern of low spatial frequency to elicit both the pink and green after effects, and no colour would be seen since the two colours are complementary and would thus cancel. Adaptation to luminance gratings at low mesopic levels has been shown to raise the threshold only for grat-

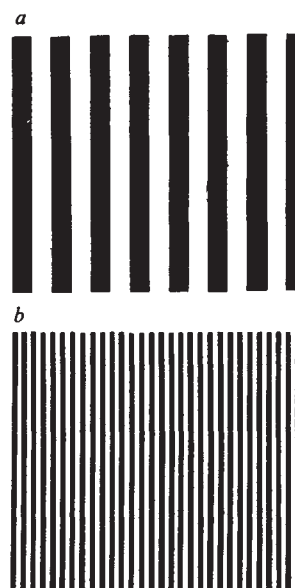


Fig. 3 Test pattern. The spatial frequencies of the test gratings were 0.5 cycles per degree (a) and 2 cycles per degree (b).

ings of similar spatial frequencies¹⁶. My results suggest that adaptation is frequency-selective at even lower scotopic levels. The existence of lateral inhibition at scotopic levels is further suggested by studies on simultaneous brightness contrast¹⁷, Mach bands¹⁸, and ganglion cells in Rhesus monkey¹⁹.

My results show that rod signals influence colour mechanisms. Rod and cone signals converge onto single ganglion cells in the monkey²⁰, and many ganglion cells have a receptive field that receives an excitatory signal from either red, green or blue cones in the centre and an inhibitory signal from a different cone type in the periphery²¹. Similar mechanisms are found in the lateral geniculate nucleus (LGN), and many of these units undergo a Purkinje shift when dark adapted, thus showing a rod input²². Ganglion and LGN cells have circular receptive fields; however, many units in the striate cortex are sensitive to line orientation and colour²³. The cortical units have receptive fields whose excitatory and inhibitory areas receive inputs from the same cone type²⁴, and thus they are highly sensitive to the spatial properties of a red or green pattern—unlike the precortical cells. Adaptation to a coloured grating might thus depress sensitivity in cortical cells tuned to the adapting colour, orientation and spatial frequency. A test pattern of the same orientation and spatial frequency would then produce less response in these units, and this imbalance may signal the complementary colour. Rod signals may also converge onto these mechanisms, and thus the colour effect may be seen at scotopic levels.

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Rhesus monkeys raised in isolation and then given access to unlimited food were found to overeat to the same degree as monkeys with hypothalamic lesions². Clinical observations suggest that the inability of some obese people to control their food intake is related to deficits in early learning experiences, with inability to differentiate between nutritional need and other signs of discomfort³. Experimental observations have confirmed that obese and normal subjects differ in their eating responses to internal and external cues⁴.

We report here evidence which casts doubt on the prevailing opinion. Using a group of baboons (*Papio* sp.) raised in the laboratory, some of whom had been fat as infants, we have been able to evaluate the relative importance for later weight regulation of excessive fat formation in infancy and of defects in the regulatory mechanisms. No adipose cell count was done during the period of infantile obesity.

The baboons were delivered by Caesarean section within a 6-week period, with birth weights ranging from 0.65 to 1.1 kg. They were fed a mixture simulating the composition of natural baboon milk, which was given by bottle at scheduled intervals in measured amounts to insure normal growth and weights⁵. This liquid diet was composed of a commercial human infant formula, SMA/S-26 (Wyeth), water and corn oil in the ratio of 90:80:1. At the end of the first month some infants were put on a self-feeding device, permitting *ad libitum* feeding; these animals gained excessive weight and became visibly fat⁶. After 7 to 8 months all animals were weaned gradually on to solid food, a commercial monkey biscuit which was approximately

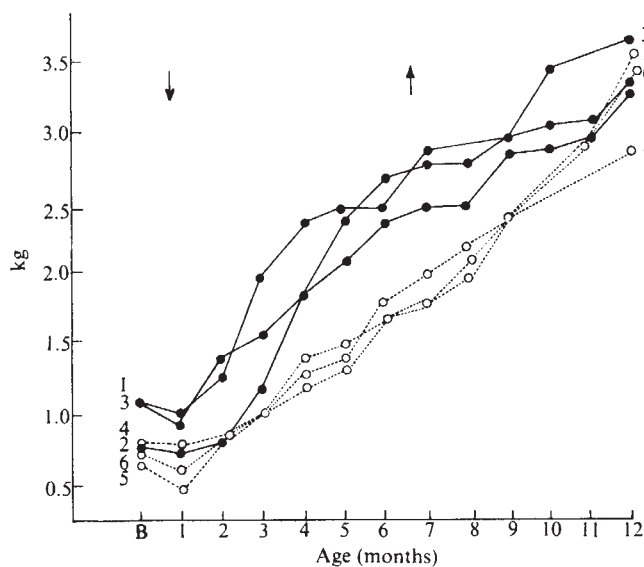


Fig. 1 Weight of baboons during infancy. The heavy lines represent three animals on a self-feeding device; the onset is indicated by the first arrow (↓). The dotted lines show the weights of three animals on measured feeding. The second arrow (↑) indicates onset of gradual weaning of all animals to solid food.

25% protein, 5% fat, 4.5% fibre and 3% mineral. The weight gain of the fat infant baboons slowed down. All animals were weighed regularly every month.

From birth until 1 month of age these animals were housed in stainless steel boxes and were then transferred to conventional stainless steel primate cages. They were kept singly but could hear and see each other in rooms which housed several baboons. They were cared for by a minimum number (two or three) of animal care personnel which remained the same during the whole period.

For our experiment we used approximately 2.5-yr-old animals (pre-puberty): three had formerly been obese, and three, which were of equal age and had not been fat as infants, served as controls. Figure 1 shows the patterns of weight gain in these

Infantile obesity and later weight control in the baboon

RECENTLY widely quoted studies imply that overfeeding in infancy is associated with increased formation of adipose cells and predisposes to obesity later in life. This has been documented for newborn rats in whom the high adipose cell count persisted after overfeeding was discontinued¹. In rats which had been fed sparsely as newborns for up to 21 d, the fat cell count did not increase when they were fed more generously later. The establishment of regulatory controls over food intake has also been related to early experiences.

Table 1 Weekly changes in weight on *ad libitum* feeding

No.	June 3	July 6	13	20	27	August 3	10	17	24	7	September 14	21	28	October 5	5	12	19	26	Nov. 2	Dec. 14
<i>Formerly fat baboons</i> (self-feeding in infancy)																				
1	7.5	7.5	+7	+1	+1	0	+3	0	+1	+2	-2	-1	+4	+1	9.2 (+22%)	-7	-1	0	-1	+2
2	6.7	6.6	+4	+4	-2	+1	+1	-1	-1	+4	+2	-1	+3	-1	7.9 (+20%)	-6	0	-1	+1	0
3	7.7	7.7	+6	0	+1	0	+2	0	+1	+3	+2	+1	+2	0	9.5 (+23%)	-6	-1	0	+1	-3
<i>Experimental controls</i> (measured feeding in infancy)																				
4	6.9	7.0	+6	0	0	+2	0	0	+1	+1	+2	0	+3	0	8.5 (+21%)	-6	-1	-2	+1	0
5	7.6	7.6	+7	+1	-1	+2	+3	-3	+2	+4	-3	+1	+1	0	9.2 (+21%)	-5	-4	+1	-1	+5
6	6.5	7.1	+3	-2	0	+4	0	-2	+2	0	0	+1	+2	0	7.9 (+12%)	-3	0	+1	+2	0

Figures are kg. *Ad libitum* feeding began on July 6 and measured feeding recommenced on October 5.

six animals during infancy. Animals on the self-feeding device had a slightly higher birth weight, 0.9 kg as average, compared with an average of 0.73 kg for the others. On the self-feeding device weight increased at an excessive rate, and reached 110% of birth weight at the end of the fourth month and 190% at the end of 6 months. The corresponding increases for the animals on measured feeding were 75% and 130% respectively. At 12 months, the weights of all animals were in the same range.

Our experiment consisted of offering an inexhaustable food supply by keeping the food containers filled with monkey biscuits at all times. Two to three times as much food was consumed by the experimental animals than by those on measured laboratory feeding. Because of the wasteful feeding habits of baboons, it was not possible to measure the exact amounts eaten. The rapid gain in weight reflects the greatly increased food intake. Animals were weighed weekly (Table 1). Total body weights during and following the experimental period are shown in Fig. 2. The overall weight gains during the 3 months of the experiment were very similar for five animals—20 to 23% of the starting weight. One animal (No. 6) who had gained weight rapidly during the preceding month,

gained only 12%. Three other baboons of similar ages who continued on the measured laboratory feeding, showed only 4% weight gain during this period.

When excess feeding was discontinued and the animals were again given measured amounts, there was rapid loss of weight during the first week and gradual loss of weight and then stabilisation during the next few months; patterns were similar for all animals.

We found that with access to an unlimited food supply, the formerly fat and nonfat animals reacted in the same way. They all ate excessively and gained weight at a similar rate. Although the immediate control over food intake seemed deficient, as had been described for monkeys raised in isolation², there seem to be limiting regulatory mechanisms for what can be assimilated, without recognisable difference in the formerly fat and nonfat animals.

The lack of difference in the rates of gain and loss between the formerly fat and nonfat animals contrasts with expectation according to reports for experimental rats on excess feeding during infancy. In baboons the later rate of weight gain, and partial retention of the excess weight, is for all practical purposes identical in formerly fat and nonfat animals. Perhaps over-feeding was not continued long enough to cause changes that would influence eating patterns and facilitate fat storage later on. This is in agreement with clinical observations. Though it is true that infantile obesity is frequently followed by life-long obesity, this occurs only when the conditions that make for over eating and obesity persist⁷.

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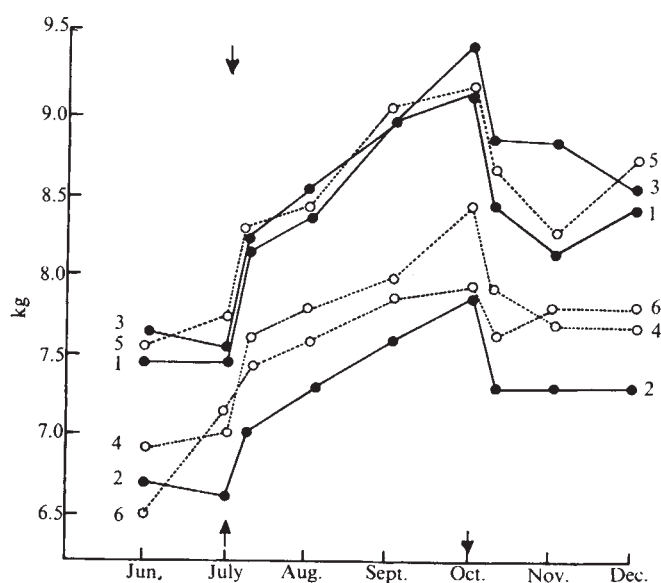


Fig. 2 Weight changes of baboons during *ad libitum* feeding. The heavy lines represent three formerly fat baboons, the dotted lines three animals who had not been fat. The first arrows (↑) indicate onset of unlimited food supply, the second arrow (↓) return to measured feeding for all animals.

Evidence from Lincolnshire of the age and intensity of the mid-Devensian temperate episode

THE study of fossil beetles from several sites has indicated a period of temperate climate in the middle of the Devensian (Weichselian)¹. Upton Warren (Worcestershire), dated at about 42,000 yr BP, yielded an assemblage composed, in part, of thermophilous beetles². At Four Ashes (Staffordshire) deposits of the same age which contain warm species overlie Devensian silts of arctic aspect³. A further warm fauna of this age has been investigated from Isleworth, Middlesex (personal communication from G. R. Coope). New faunal and ¹⁴C evidence from this critical period of mid-glacial climatic change is currently being investigated from Tattershall Castle Pit, Lincolnshire (map ref. TF570210).

At this pit organic horizons of several ages lie in sands and gravels above a till rich in Jurassic and Cretaceous erratics. The lowest organic deposit, a shell marl containing a terrestrial snail fauna characteristic of open conditions, may be of Upper Wolstonian age (personal communication from J. G. Evans). This is overlain by compact woody peat assigned on pollen evidence to subzone Ip IIB of the Ipswichian interglacial (personal communication from L. Phillips). An extensive beetle fauna extracted from this peat includes non-British beetles such as *Brachytemnus submuricatus* Schön. and *Valgus hemipterus* L. previously recorded from interglacial deposits^{4,5} and *Isorhipis melasoides* Lap. and *Pycnomerus terebrans* Ol., species noted in Postglacial deposits^{6,7} but now extinct in Britain⁸. Wood-boring genera such as Cerambycidae and Scolytidae are well represented and species of Anobiidae include *Xestobium rufovillosum* (Deg.), *Anobium fulvicorne* Sturm and *A. punctatum* (Deg.). Examples of other tree-dependent species, *Melasis buprestoides* (L.), *Dryophthorus corticalis* Pk., the oak leaf-miner *Rhynchaenus quercus* (L.) and the predators *Colydium elongatum* (F.) and *Dasytes plumbeus* (Müll.), demonstrate the presence of mature, mainly deciduous forest. Other environmental factors—rich humic soils, areas of marshy vegetation, influent streams and in particular, warmer summer temperatures than in South England today—are indicated by this interglacial fauna.

Above this peat are two mid-Devensian organic silt horizons. The lower of these, which occurs within frost-disturbed gravels or directly on the peat, has ¹⁴C ages of 42,100 $\pm^{+1400}_{-1100}$ yr B.P. (Birm 309) and 44,300 $\pm^{+1600}_{-1300}$ yr BP (Birm 408), and it has yielded a cold, impoverished fauna. Characteristic foreign species include *Diachila arctica* Gyll., *D. polita* Fald. and *Dyschirius septentrionum* Munst. (Carabidae), *Helophorus obscurus* Popp. and *Ochthebius kaninensis* Popp.⁹ (Hydrophilidae), *Olophrum boreale* Pk., *Boreaphilus henningianus* Sahlb. and *B. nordenskiöldi* Pk. (Staphylinidae). These are now largely confined to areas of high latitude or extreme continentality. Several other species are now restricted to Northern Britain. This fauna indicates tundra conditions, with tracts of sterile soil between low plants and ephemeral ponds.

Stratigraphically above and thus demonstrably younger than this 'arctic' silt is a more continuous horizon which has yielded a remarkably thermophilous fauna. Its ¹⁴C ages of 43,000 $\pm^{+1400}_{-1100}$ yr B.P. (Birm 341) and 42,000 $\pm 1,000$ yr BP (Birm 409), apparently overlapping those of the 'arctic' silt, underline the closeness in age of the two horizons, despite their marked faunal contrast. Noticeable among the several hundred species which make up the warm assemblage are certain non-British beetles such as *Hydrochus flavipennis* Kust. (R. B. Angus, unpublished work) which is today found in Southern Europe and *Aphodius bonvouloiri* Har., now found only in Spain. *Pseudocleonus cinnereus* Schrank and *Chrysolina limbata*

L. are Central and Southern European species, and *Helophorus discrepans* Rey lives in East Europe and at high altitudes in Spain. A high proportion of the species today found in Britain, including *Nebria livida* (L.), *Porcinolus murinus* (F.), *Crypticus quisquilius* (L.) and *Otiorrhynchus ligustici* (L.) occur only in the southern half of the country. Clearly this assemblage requires average July temperatures at least as high as those of South England. Despite the occurrence of certain East European species, there is no evidence for marked continentality and it is unlikely that the total fauna would survive particularly low winter temperatures.

The duration of this warm, mid-glacial episode was short. A cold, continental fauna from deposits of 39,300 $\pm^{+1350}_{-1160}$ BP (Birm 333) at Queensford, Oxfordshire (G. R. Coope personal communication) indicates that a considerable degree of cooling had occurred by this time. At Tattershall the reduction in the numbers of thermophiles in successively younger samples from this horizon may be the result of falling temperatures. The structure and ecological requirements of this interstadial fauna provides further evidence of the shortness of this climatic oscillation; this may be illustrated by comparison with the earlier interglacial fauna. Both assemblages indicate warm conditions at the time they were deposited, but the mature, deciduous forest habitat of many of the interglacial beetles obviously developed during a long period of climatic stability. Such vegetation maturity is not demanded by the interstadial fauna which is largely composed of species that feed on low plants and grasses, as well as abundant Scarabaeidae associated with the large mammal population which grazed the area. This insect fauna, able to respond rapidly to climatic change by colonising a newly favourable area⁴, lacks any species whose habitats require a long period of development. In particular, tree-dependent species are absent although temperatures were high enough to permit forest growth. This fact, while dispelling any idea that this fauna is interglacial, supports the stratigraphical and radiocarbon evidence of its mid-glacial age.

I thank Dr R. B. Angus, Dr G. R. Coope and Mr P. J. Osborne for help with this project, and Professor F. W. Shotton for reading this manuscript. This research is being undertaken during the tenure of a Natural Environment Research Council grant.

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⁷ Buckland, P. C., and Kenward, H. K., *Nature*, **241**, 405–406 (1973).

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book reviews

Second generation environmentalists

Environmental Issues: Population, Pollution and Economics. By Lawrence G. Hines. Pp. ix+339. (Norton: New York, December 1973.) \$9.75 cloth; \$3.25 paper.

Society and the Assessment of Technology: Premises, Concepts, Methodology, Experiments, Areas of Application. By F. Hetman. Pp. 420 (OECD: Paris; HMSO: London, 1973.) 38 francs; £3.36; \$9.50.

NEITHER of these books is an original contribution to knowledge, but they are nevertheless both very useful. They reflect the extent to which environmental concerns and technology assessment have become part of normal policy making and analysis. The typical early literature of this genre had an evangelistic and strong emotional tone, which was often conveyed in the title as well as the text. One thinks, for example, of *Our Plundered Planet*, *Silent Spring* and the *Population Bomb*.

Both of these books belong emphatically to the second generation. Not that they are lacking in concern; Hines in particular makes quite explicit the strength of his attachment to the preservation of natural amenity and wilderness. But both are very much alive to the complexity of the issues and the trade-offs which are inevitably involved. This means that they are rather duller books than the best of the pioneering single-minded first generation advocacy; but as against this they are much more realistic and much more acceptable to administrators.

The scope of Hines's book is wider than that of Hetman's in one sense: its first section is devoted to the world population and resource problem brought into the limelight by the MIT "World Models". His sceptical critique of the MIT work, although much less thorough than that of the Sussex Science Policy Research Unit, comes to similar conclusions. The second part deals with problems of cost/benefit analysis and the third part with policies to combat pollution. Hetman's book does not discuss the "Limits to Growth" problem at all, nor does it discuss specific policies in relation to pollution. It is devoted entirely to a critical review of the various techniques of 'technology assessment' of which 'cost-benefit' is treated as one example.

Although there are important similarities,

the books differ greatly in style and presentation. Hines's book is far better written, and does not suffer from poor translation into the curiously effete and de-humanised English jargon of the international bureaucracies. The Hines book is far better produced and edited, and the diagrams, tables and references are of a standard to which we have become accustomed in the best American college textbooks. The OECD publication by contrast is rather poorly produced and many of the tables are clumsy. Some of them (for example, Tables 15 and 16) are simply long quotations from other sources.

This is a misfortune as Hetman's book has some outstanding merits. It manages to synthesise a great deal of information about the burgeoning technology assessment movement. It is very balanced in its own assessment of the various techniques and sensible in its awareness of their strengths and limitations. It avoids the temptation of staking everything on one particular technique—a fault which bedevils so much of this literature. Finally it recognises the importance of the political process in relation to any technique or combination of them. In its all-round grasp of a very complex problem the book compares very favourably with much of the American technology assessment literature. It is therefore a thousand pities that the book is so badly edited and produced. It is too long, often repetitive and sometimes unreadable. The two greatest faults are first: the inclusion of large chunks of material (especially in chapter 3) which should have been relegated to an appendix or the (otherwise excellent) reference list. Second, the failure to bring the book to life by thorough discussion of some real-life cases such as the third London airport or supersonic transport. Many examples are cited, but the form of citation is long enough to be boring but not thorough enough to give the full flavour and excitement of a real political problem. Perhaps these faults may be partly attributed to the inevitable limitations of publication through an international organisation, but the OECD has sometimes been outstanding in its toleration of lively and critical material (even when it was embarrassing to member governments) and in the quality of its publications.

By contrast, one of the best chapters in the Hines book is on the "Middle Snake River Hydro-electric Project". This makes the essential points far better than any theoretical analysis, since it shows so clearly just how cost-benefit analysis was manipulated in practice to serve particular interest groups, and the real practical difficulties involved in the attempt to put a market valuation on some of the intangibles. Hines is a competent professional economist but he has taken the trouble to learn a good deal about water and air pollution, and he therefore makes a modest success of his ambitious goal to transcend the normal disciplinary boundaries of economics. One must hope that this approach will become a new fashion in economics, either through interdisciplinary teams or by the individual efforts of men like Hines. He explains in a lucid, albeit very elementary manner, the failure of the market mechanism in decisions about the environment, and the inevitability of public responsibility and policy. In this respect, of course, his book is a welcome return to an old fashion: the original classical discipline was political economy.

C. FREEMAN

Tetrahymena

Biology of Tetrahymena. Edited by Alfred M. Elliott. Pp. x+508 (Dowden, Hutchinson and Ross: Stroudsburg, Pennsylvania; Wiley: Chichester, December 1973.) £19.25.

OF all the protozoa *Tetrahymena* is probably the most studied genus. *T. pyriformis* (formerly called *Glaucoma pyriformis*), which occurs in ponds and streams the world over, was first successfully cultivated axenically, that is, in the absence of the other organisms which comprise its normal diet, in 1923 by André Lwoff. By 1951 Kidder and Dewey had succeeded in growing it in a chemically defined, though very complex, medium. Later developments have been the discovery by Elliott and his colleagues of mating types of *Tetrahymena*, following similar work in *Paramecium*, thus opening up the possibilities of doing genetic work, and these have been exploited by Nanney, Allen and others. On technical grounds, however, *Tetrahymena* has turned out to be rather less suited to

from abacus,
through...bubble chamber...
carposporangium...
flying tuck...
pentaerythritol tetranitrate...
and (sic)
uncertainty principle...
to zymetic

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genetic experiments than *Paramecium*, though for biochemical work *Tetrahymena* is far superior. Finally *Tetrahymena* has recently been used for developmental studies, especially of the elaborate surface patterns of basal bodies, cilia, oral apparatus and so on. Thus the organism is now available for many different kinds of work, and a general review of the current state of knowledge about it would be very valuable.

This volume consists of some thirteen chapters written by about as many specialists and ranges over the taxonomy, cytology, life cycle, biochemistry, genetics and cortical development of the organism, with a final chapter on *Tetrahymena* as a nutritional and pharmacological tool. The styles of the different chapters are very diverse, from the relatively prosaic descriptive accounts of the taxonomy, life cycle, and some of the biochemistry, to far flung speculations in the genetics chapter of Allen and Gibson. Little attempt seems to have been made to interrelate the material in different chapters, and there is a certain amount of overlapping. The book is really a series of separate articles, tied together only by the fact that the same organism is mentioned in all of them. Viewed in this way, it is a useful source of information about the state of knowledge of various special topics in 1972

or slightly earlier, and for anyone planning to use the organism experimentally, it is essential reading.

The chapter by Frankel and Williams on "Cortical Development" is very comprehensive and clear, and the book ends with a bibliography prepared by Dr Elliott, after consultation with John Corliss, of no fewer than 1,700 citations stated to comprise all major and most minor publications on *Tetrahymena* up till 1972. This is one of the most valuable features of the book.

It seems to me that research on *Tetrahymena* may be approached from two opposing viewpoints. The first involves using the organism as a model cell whose behaviour resembles closely enough that of higher animals to enable it to be used for trials of pharmacological and dietary substances intended for use on higher animals or man. This is discussed in the final chapter by Hutner and others. It seems that one should be cautious in accepting the results of such trials, especially of drugs suspected of having carcinogenic, toxic, or other harmful effects on human cells, though the use of *Tetrahymena* as an agent for assaying preparations of particular amino acids or vitamins which are an essential requirement in its diet is more reliable.

The second approach to research on *Tetrahymena* is to study it for itself, as a remarkably interesting biological system, with many technical advantages for experimental work, helping us to understand all sorts of biological processes, whether biochemical, genetic or developmental. Here it does not matter so much that *Tetrahymena* differs markedly from cells of higher animals, for example in its presence of two kinds of nuclei, its extraordinarily elaborate surface structures, and finally its 'protozoan' nature—compressing all life processes into one and the same compartment.

Biologists expert in other matters, or those with more general interests, would be disappointed to find in this volume so little effort at assessing the overall importance of the work, and no attempt at all to write a single coherent account.

G. H. BEALE

Resistant plants

Breeding Plants for Disease Resistance: Concepts and Applications. Edited by R. R. Nelson. Pp. xii+401. (Pennsylvania State University: University Park and London, November 1973.) £6.85.

PLANT diseases are major factors limiting agricultural production throughout the world. Resistant varieties, which are less damaged by disease than others, are widely used to control diseases. This book attempts to summarise the

present state of our knowledge concerning the production of resistant varieties, with particular reference to some of the most economically important crop species.

The book is divided into two main parts. In part 1 the editor gives his views (many of which he admits are controversial) on the concepts, principles and terminology of breeding for disease resistance. Part 2 consists of sixteen chapters in each of which work on breeding for resistance in a particular crop species is summarised. Each chapter has been written by specialist pathologists, geneticists and plant breeders, mainly from the United States, with research experience in that crop. The format of these chapters varies from crop to crop but generally involves a short introductory section on the origin, economic importance and breeding system of the crop concerned and a description of the main sources of disease resistance. This is followed by accounts, which are often extremely brief, of breeding for resistance to some of the main virus and fungus diseases of the crop. An example of this brevity is afforded by the chapter on disease resistance in peas in which there are subsections on eighteen diseases in less than seventeen pages. Conversely, the chapter on soybeans deals in much greater detail with only four diseases in fourteen pages.

This lack of uniformity of treatment in different chapters can be disconcerting but is perhaps to be expected in such a compilation by so many authors, each with a different style and approach. It would have been useful if the editor had written a third part in which he attempted to draw general conclusions from the experiences described in the crop chapters, particularly in relation to the theoretical considerations outlined in part 1. As it is, the reader is left to draw his own conclusions from work on sixteen different crop species.

I find it surprising that a book of this nature includes no line drawings or photographs. Illustrations of disease symptoms and of examples of differences between resistant and susceptible varieties after exposure to infection would have been particularly interesting and useful. As a result of editorial policy, the book contains remarkably few references to original published work on disease resistance. A less restricted bibliography would have made this a much more useful reference book.

In spite of its limitations and shortcomings, this book is an up-to-date, authoritative account of breeding for resistance to diseases. As such it deserves to be read by all those concerned with plant pathology and plant breeding.

G. E. RUSSELL

Ions into solids

Ion Implantation. By G. Dearnley, J. H. Freeman, R. S. Nelson, and J. Stephen. P. xv+802. (Defects in Crystalline Solids, vol. 8.) (North-Holland: Amsterdam and London; Elsevier: New York, 1973.) Dfl. 225; \$79.

EVER since it was found possible to generate beams of energetic ions, investigators have been directing them into solids, and noting the effects. As expected, a set of complex but interesting phenomena was to be found in the interaction of the beam with the solid; moreover, it was soon realised that implantation was a useful method of modifying solids, while ion channelling was a unique diagnostic tool. A mushroom of literature on the subject developed in the late 1960s.

An anticipatory thrill is thus in order when one opens a book in which four authorities at a centre like AERE, Harwell, have combined to try and review the field, especially when the weight and thickness of the book indicate that the coverage is likely to be broad and the treatment detailed. The main question then is not of accuracy or authority—these are taken for granted—but whether the authors have done their reading public the maximum service in digesting and explaining this great expanse of literature as well as bringing out salient points of their own research. My impression as a user of ion-implanted specimens is that they have certainly made a very good attempt at dealing at some depth with the physics and engineering aspects which relate to the uses of implantation. What failures there are will be felt more by those looking for insight and some are discussed later.

The book consists of four main sections, each attributed to one of the authors, followed by a shorter chapter on the less usual applications. Apart from the unavoidable lack of unity which this method gives, the sections have been well coordinated as to subject matter, level of discussion, graphics and so on, although there is little cross referencing. Only a few things fall between the cracks; for example all of the authors are mainly interested in ion implantation into metals or semiconductors, so that insulators are given very little space. The fact that each author is treating his special field also means that he writes at a rather high intellectual level and pace and (with the exceptions described below) rarely catches his breath to explain to lesser mortals what the inwardness of some statement may be. For example, one author remarks that metals, unlike semiconductors, remain crystalline under bombardment even up to several hundred

displacements per atom, and that the reason for this fundamental division in ion-bombardment effects is not known. Surely, between the four authors, is not some little speculation on the reasons desirable? One can certainly form some hypothesis on the question in terms of the kinetics and type of vacancy/interstitial recombination available in the two forms of solids. Indeed, throughout, few panoramic views of the subject are essayed.

The author of the first main section, on ion penetration and channelling, is both the most concise and the most willing to comment intelligently on the meaning and prospects of the research work discussed. For example, the very useful phenomenon of radiation-enhanced impurity diffusion is made more than usually fascinating by useful asides. The discussion of channelling is, as expected, masterly. The next section, on the physical state of ion-implanted solids, is also a good exercise in compression; perhaps too compressed, since some omissions can be felt. Here was the place for a discussion of the annealing of clustered defects, with all the physical insight which this technique gives. The annealing of metals, bombarded, say, with deuterons at low temperature, yields an important insight into ion damage. Yet the whole topic of annealing of metals is dismissed in one page. This author indeed explicitly limited his writing to the effects observed from irradiation at or above room temperature, and this artificial barrier itself depreciates the value of the section in giving physical insight into the structure of implanted solids.

The third section is a detailed description of the art of implanting. Ion sources, analysers, accelerators and targets are described by a past master of the art and a tabular appendix lists the practical needs of each element in the periodic table. This section is a handbook in itself, unique in that many engineering aspects of ion beam production are only to be found in theses or specialist reports. With its high content of unpublished Harwell data, it probably merits its 200 pages but its length, of course, contributes to the horrendous price of the book.

The fourth part is of similar length, and is on the use of implantation in semiconductor device fabrication. This application has dominated all others to date and it can be claimed that a comprehensive review here is justified on those grounds. But the argument of rarity, used above, does not apply in this case. Should this section perhaps have been clipped to 120 pages? Coming after many published research reviews, it probably should have; however, in itself, this section is still a very well informed and complete ac-

count and can be thought of as the United Kingdom's brief on the subject of implantation in silicon. The final chapter, on other applications of implantation, is a useful compilation.

When a work so significant appears at such a price the reviewer must also try to advise librarians as to whether it is a good buy. I have already told my librarian that it is, and to others would say that, like Harris tweed, the price may hurt but the goods will give long and unwilling service.

A. G. HOLMES-SIEDLE

Nature and nurture

Introduction to Behavioral Genetics. By G. E. McClearn and J. C. Defries. Pp. 349. (Freeman: San Francisco, 1973.) \$10.

SOME of the most important developments in the interdisciplinary field of behaviour genetics have occurred in the thirteen years since Fuller and Thompson's *Behavior Genetics* provided the first and, until now, the only comprehensive coverage of the subject. The publication of *Introduction to Behavioral Genetics* under the joint authorship of a geneticist and a psychologist is, therefore, particularly welcome.

The introduction puts the emergence of the central problem of behaviour genetics, the nature-nurture controversy into historical perspective and the final chapter reviews the current status of this controversy as it relates to human behavioural differences that contribute to social problems. In the intervening pages the basic principles of genetics are introduced and these are elaborated as necessary throughout the rest of the book. A brief account of basic statistical principles that stops short of the analysis of variance precedes the presentation of examples from the behavioural genetics literature. These are grouped under a number of headings according to whether one or many genes are involved, whether experimental breeding or cytological observation were the means of investigation, and whether the main interest of the results obtained relates to the biochemistry of gene action, the processes of development or the forces acting on natural populations. A chapter wholly devoted to quantitative genetics, which in content and approach owes much to Falconer's *Introduction to Quantitative Genetics* reflects the present day emphasis on quantitative differences in behaviour.

Though the authors can rightly claim to have produced an introduction to the entire range of behaviour genetics, they have done so, to some extent, by offering *ad hoc* solutions to the immediate issues raised by the

behavioural examples rather than using well-chosen examples to develop and illustrate the experimental methodology and the principles underlying the analytical procedures and interpretations. It is difficult to believe that more appropriate examples could not have been found or that they could not have been used more effectively to illustrate the principles of experimental design, model building, hypothesis testing, interpretation and prediction. These are, however, relatively minor deficiencies compared with the advantages of having a concise, up to date account of the subject.

J. L. JINKS

Deformed embryos

Environment and Birth Defects. By James G. Wilson. Pp. xiv+305. (Environmental Studies: an Interdisciplinary Monograph Series.) (Academic: New York and London, December 1973.) £9.10.

THOUGH mortality at all ages in the period of childhood and adolescence has fallen dramatically during the past few decades mortality from congenital defects has shown little change. The relative importance of congenital defects has increased and continues to increase. This applies both to mortality and morbidity. Knowledge on the aetiology and underlying pathogenesis of congenital defects is very limited and much of the information available is widely dispersed in the literature. A book which brings together and unifies much of the existing information on teratology—defined as the adverse effect of environment on developing systems—is to be welcomed and fills a gap in the medical and biological literature.

The book begins by identifying the broad issues in teratology including the basis on which the conceptus becomes susceptible to adverse effects; the disturbance of cellular tissue and developmental processes which constitute the basic pathology of teratology; the importance of accessibility of an agent to the conceptus in determining possible teratogenic effects; dose effects; and the possible range of manifestations of deviant development. The causes of developmental anomalies are discussed in terms of genetic influences, chromosomal aberrations, radiation, chemicals and drugs, dietary imbalance, infections, respiratory gas levels, extremes of temperature, metabolic or endocrine imbalance, physical trauma, mechanical factors, placental factors, antibodies and combined effects. A review of the various drugs known or suspected to cause congenital malformations (not entirely complete) is relevant to clinical pharmacology and it touches on the

possible teratogenic effects of environmental chemicals used in industry and in agriculture.

The book then goes on to discuss the means by which developmental defects occur—the mechanisms of teratogenesis—under the headings of mutation; chromosomal non-disjunction and breaks; mitotic interference; altered nucleic acid integrity or function; deficiency of precursors, substrate and enzymes; altered energy sources; enzyme inhibition; osmolar imbalance; changed membrane characteristics; excessive cell death; reduced cell proliferation; disturbed interaction between cells; and reduced migratory movement of cells. Figures are given for the general incidence of developmental defects in man based on differing assessments made on embryos, stillbirths, neonatal deaths and later deaths. The effect of teratogenic processes is classified into four categories, namely, intra-uterine death, malformation, growth retardation or functional defect.

There is a useful account of the early developmental stages of the human ovary and foetus with pictorial representation of 23 different stages from fertilisation to the 53rd day of gestation, and a discussion on the concept of critical periods of susceptibility.

Further chapters deal more specifically with the access of teratogenic agents to the foetus and the foetal defence mechanism against them, and with the means of detection of teratogenic effects in man including recognition based on observation of clusters of cases, epidemiological surveys and surveys of high risk populations.

The last quarter of the book deals largely with the contribution of animal experiments to the assessment of human teratogenic risk and to the problems which arise from such experiments. The latter include the differing effect caused by the timing of any insult, dose effect in respect of quantity of teratogen and duration of administration, the masking of teratogenesis by embryolethal effect and standards of recognition of defects. There is a section on the various animals which can be used in teratology testing. The author puts forward a suggested scheme of teratogenic testing based on the graded use of animals of different availability and cost. Lastly there are special sections on appropriate literature relative to a number of individual animals and there is a valuable general bibliography.

This book ranges over the field of teratogenic response to the environment as revealed by animals and human studies. It concentrates primarily on the broader biological issues of teratogenesis approaching the subject from a morphological rather than a biochemical standpoint. The principles of

teratological testing are discussed and the author's own recommendations regarding his scheme for animal testing of suspect drugs provides a special component to the discourse. The book is clearly and concisely written and will prove a very valuable source of information to a wide range of those who are concerned with the environment and its possible effect on human development. These would include environmental scientists, hygienists, developmental biologists, obstetricians and paediatricians, toxicologists and pharmacologists. JOHN O. FORFAR

Mathematical brainwaves

Automation of Clinical Electroencephalography. Edited by Peter Kellaway and Ingemar Petersén. Pp. viii+318. (Raven: New York; North-Holland: Amsterdam, 1973.) \$22.70; Dfl.59.

THIS book provides an excellent guide to the quantitative methods of analysis currently being employed in EEG research. In the opening chapters, the editors draw attention to the major problem involved, namely that of defining the standard method of visual analysis in mathematical terms. For the non-clinician, a comprehensive description of standard EEG analysis is included, together with examples of typical wave forms and commoner abnormalities.

The case is presented for regional centres for automatic EEG analysis, so that research methods described can be widely applied to clinical practice. To implement this programme, a telemetry system capable of carrying at least eight channels of information would be required, a suitable system operating by public telephone being described later in the book. Continuing this theme, methods are described enabling multichannel EEG data to be presented in a simplified form on a single page, either by bar graphs for the principal frequency bands (delta, theta, alpha, beta), or by compressed spectral array, neither technique requiring as experienced an interpreter as conventional paper records. Extensive data reduction is involved, and although the two methods are complementary, they are suitable only as screening tests, and not as replacements for conventional EEG techniques. The method and application of Fast Fourier Transform are considered, and among many other topics included are pattern recognition techniques and EEG analysis in sleep.

The book is well presented and clearly illustrated throughout, and should prove a valuable reference work for those with interests in this expanding field.

L. M. BRANCH